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DETERMINANTS AND CONSEQUENCES OF THE PHARMACOKINETICS OF RIFAMPICIN, ISONIAZID, PYRAZINAMIDE AND ETHAMBUTOL IN A COHORT OF TUBERCULOSIS PATIENTS

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ABSTRACT

A prospective pharmacokinetic study was conducted amongst a cohort of 142 patients with tuberculosis (TB) susceptible to rifampicin and isoniazid at Brewelskloof Hospital, Worcester, in the Western Cape.

The pharmacokinetic profiles of rifampicin, isoniazid, pyrazinamide and ethambutol in plasma were derived from blood samples taken within 1 hour before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 hours after drug administration on a single occasion, 2 months after admission to the hospital. Noncompartmental methods were used to estimate the peak concentration (Cmax), the time to reach peak the concentration (Tmax), the area-under-the-curve from 0 to 8 hours (AUCt), the area-under-the-curve from 0 to infinity (AUCi), the half-life and the elimination rate constant (k), for each drug. The association of the pharmacokinetic measures with patient and treatment factors (including age, sex, body mass index, acetylator type, HIV-infection status, drug dose/kg, drug formulation characteristics, chemistry and haematology profiles, and history of prior antituberculosis treatment, drinking and smoking habits) were described using multiple linear and logistic regression methods. Treatment response was measured by proportional weight gain over 2 months from the time of admission, sputum smear and culture at 2 months after admission, the treatment outcome recorded in the TB-registers, and the rate of treatment failure, death or relapse from 2 until 24 months after admission to the hospital. Determinants of treatment response were identified by regression of patient factors (including correlates of immunity, demographic, clinical and laboratory characteristics) and treatment factors (including antituberculosis drug levels and formulation factors) on the markers of treatment response.

Low and variable rifampicin levels were prevalent amongst this patient population. HIV-infection and formulation characteristics, amongst others, were important determinants of the respective pharmacokinetic measures of the drugs. Rifampicin and ethambutol levels were significant determinants of sputum conversion at 2 months; increased ethambutol concentrations and high doses of pyrazinamide were associated with reduced weight gain during the first 2 months of hospital admission; and higher peak concentrations of isoniazid were found in those with worse outcomes in the TB-registers. The prognosis at 24 months was predominantly determined by immunity. More vigorous whole blood interferon-gamma responses to PPD stimulation at 2 months after admission to the hospital were associated with substantial reductions in the risk of treatment failure, death or relapse. Higher creatinine levels and absence of HIV-infection were also had important protective effects.

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ABBREVIATIONS

ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
AUCt	area under the concentration-time curve from 0 to 8 hours after drug administration
AUCi	area under the concentration-time curve from this time of drug administration until infinity
BMI	body mass index
CI	confidence interval
Cl F obs	total body clearance
Cmax	Maximum (peak) drug concentration
EDTA	ethylene diamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FDC	fixed dose combination
γ-GT	gamma glutamyl transferase
GSH	Groote Schuur Hospital
HIV	human immunodeficiency virus
HLA	human lymphocyte antigen
HPLC	high performance liquid chromatogaphy
IOV	interoccasional variability (intraindividual variability)
IUATLD	International Union Against Tuberculosis and Lung Disease
K/ke/ λ_z	elimination rate constant
LC-MS	liquid chromatography - mass spectroscopy
MCV	mean cell volume
NHLS	National Health Laboratory Service
PK	pharmacokinetic
PPD	purified protein derivative
SAIMR	South African Institute for Medical Research
SD	formulations containing a single drug only
TB	tuberculosis
UCT	University of Cape Town
UN	United Nations
Vz F obs	volume of distribution
WCC	white blood cell count
WHO	World Health Organization

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INTRODUCTION

The project described in this thesis was initiated in 1999 in response to the request by clinicians at Brewelskloof Hospital to investigate whether low drug concentrations could be responsible for the poor response to fully supervised treatment of certain individuals who had drug-sensitive tuberculosis. The study aimed to address several issues unresolved in the literature at the time and outlined below, including: the normal patient ranges for pharmacokinetic measures of the first-line antituberculosis drugs; the identification of patients at risk of low drug concentrations; the identification of risk factors associated with low drug concentrations; the role of drug concentrations in determining the response to treatment and treatment outcome.

Project objectives

(i) To measure the concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol in a large sample of the patient population at Brewelskloof TB Hospital on the outskirts of Worcester, Western Cape. Relatively intensive sampling was used over an 8 hour time period following administration of the patients' routinely prescribed medicines. The pharmacokinetic profile for each drug was obtained in each patient on a single occasion after 2 months of antituberculosis therapy in the hospital.
(ii) To describe the normal ranges for the pharmacokinetic measures for each of the drugs in this patient population.
(iii) To define efficient methods for identification of patients with low drug concentrations.
(iv) To measure patient and formulation characteristics in this patient population and describe the risk of low drug concentrations associated with these factors.
(v) To measure the response to standard treatment regimens for drug sensitive tuberculosis in this patient population. Data was measured at 2 months after admission to hospital and outcome data were gathered until 2 years after admission to the hospital.
(vi) To establish whether low drug concentrations are associated with a poor early response to treatment (measured at 2 months after admission to the hospital), or an increased risk of disease relapse (up to 2 years after admission to the hospital) in this patient population.
(vii) To measure cytokine markers of immunity in this population and the relationship of these markers to treatment response.
(viii) To measure the susceptibility of the infecting strains of *M. tuberculosis* in this population to isoniazid and rifampicin and the relationship of drug susceptibility to treatment response.

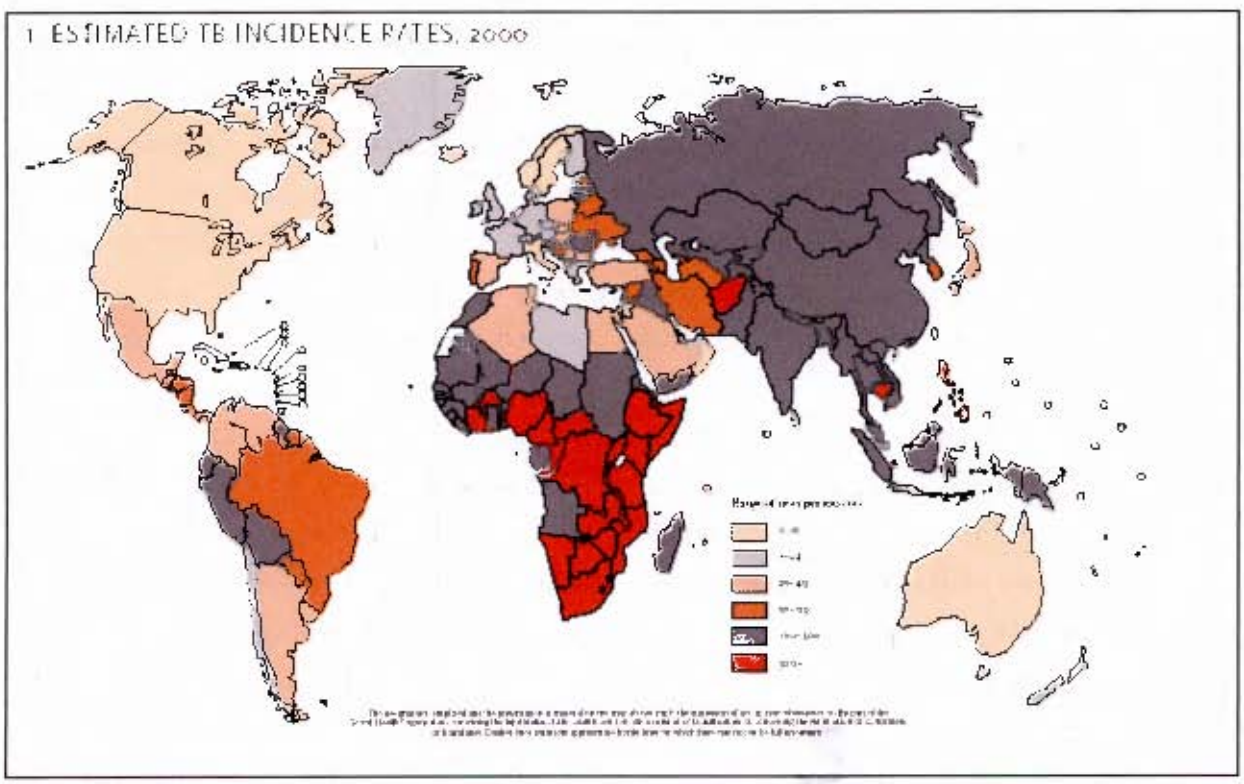
Background information and literature review

The global and regional impact of tuberculosis

In contrast to the generally declining rates of communicable diseases, the global burden of tuberculosis is increasing[1,2] (The Global Burden of Disease). Young children and the economically active age group are prominently affected[3]. While global initiatives are dominated by international finance and trade, the launch of the Global Fund for AIDS, Tuberculosis and Malaria by the UN (2002) is evidence that the global importance of this threat to public goods (including health) is recognized[4].

The worldwide incidence of new tuberculosis (TB) cases in 2002 was estimated to be 8.8 million (141/100 000)[2]. The incidence rates are highest in Africa (350/100 000 in 2002, with a 7% annual increase in the number of cases, in countries with a high burden of HIV-infection) and Sub Saharan Africa bears the brunt of this epidemic (figure 1).

Figure 1. The global distribution of tuberculosis (from: World Health Organization. Global Tuberculosis Control: Surveillance, planning, financing. WHO Report 2002. Geneva, Switzerland. WHO/CDS/TB/2002.295)



In South Africa there were approximately 558 new cases of TB per 100 000 of the population in 2002[2]. The country is ranked ninth for the total number of cases, and the costs of TB control in South Africa were estimated to be about US$ 300 million in 2003[5].

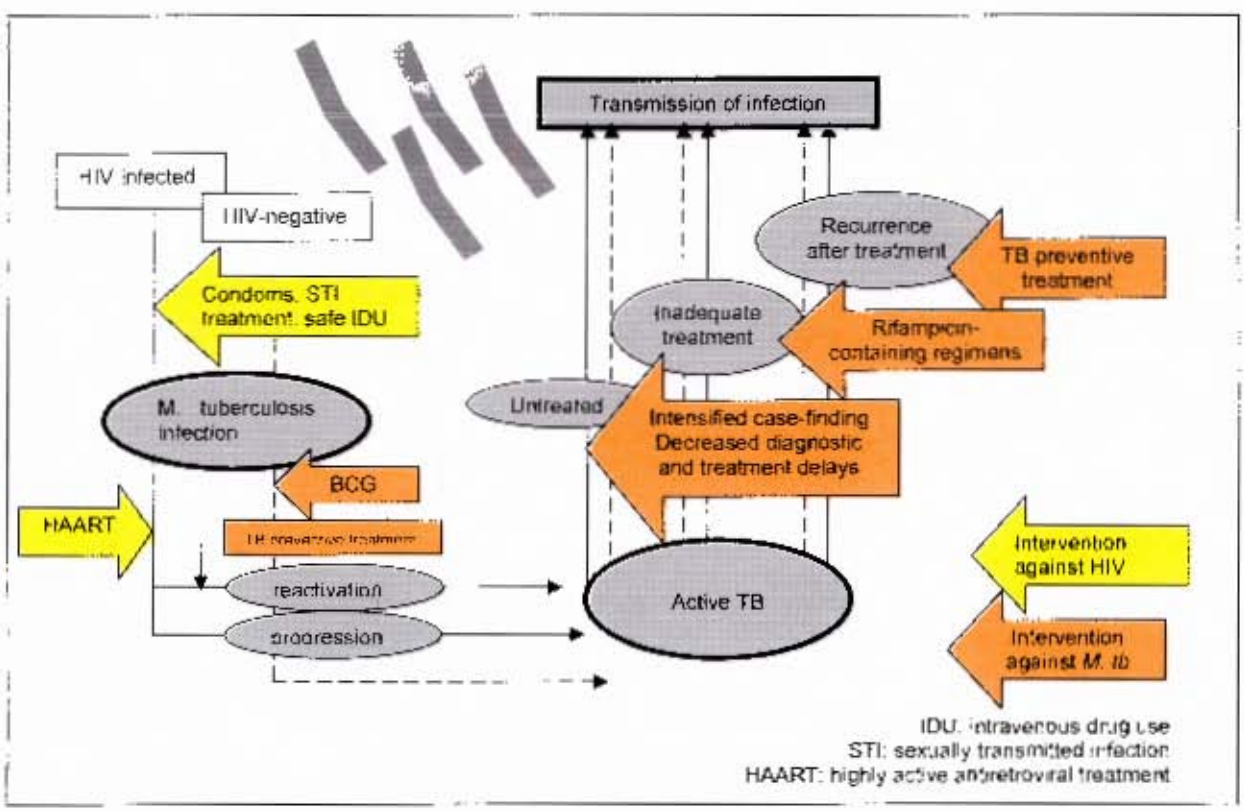
There is a particularly high incidence of tuberculosis in the Western Cape province: 932/100 000 in 2003 [4]; and the rates in the Boland/Overberg region are higher than those recorded for the province as a whole (in 2001 the number of new smear positive cases in the Boland/Overberg region was 36% above the provincial number)[5].

The especially high prevalence of tuberculosis in the Western Cape is fuelled by social conditions of inequality and poverty. High levels of unemployment, overcrowding, alcohol consumption, smoking, malnutrition and dietary deficiency of vitamin D have been implicated as risk factors for susceptibility to tuberculosis; vitamin A and zinc and may also be important micronutrients for resistance to tuberculosis[7-10]. High incidence rates in children and a high proportion of recurrent cases caused by recent transmission of new *M. tuberculosis* strains (as opposed to reactivation of disease by the original strain), are evidence that high rates of transmission amplify the disease burden in high prevalence areas[11]. Casual transmission is believed to be an important mode of disease transmission under these conditions[12,13]. The virulence characteristics of the prevalent strains of tuberculosis may contribute to the disease burden; the putatively easily transmitted and relatively virulent Beijing like strain, 129, is prevalent in at least one Western Cape community[14]. Genetic variations are also determinants of susceptibility to tuberculosis (eg. variations in the NRAMP1 gene - the natural resistance associated macrophage protein gene[15], certain HLA alleles, interleukin-1 and the vitamin D receptor; and altered production of interferon-γ, the interferon γ receptor, tumour necrosis factor-α and interleukin-12 receptor β1[16]), but patterns of genetic susceptibility have not been identified in Western Cape populations as yet. Furthermore, environmental factors such as prior exposure to other pathogens may influence the type and efficacy of the immune response to tuberculosis[17].

HIV (human immunodeficiency virus) is the most potent force driving the tuberculosis epidemic. In Africa 31% of new adult cases were attributed to HIV-infection in 2000[18]. While the worldwide prevalence of TB HIV co-infection in adults was 0.42% in 2000; in South Africa (and 8 other African countries) the co-infection rates approached 5% of the population[5]; in some districts of Cape Town, HIV and TB co infection rates are alleged to be as high as 35 40%. In South Africa, 60% of tuberculosis patients have HIV infection[16,18]. The size of the tuberculosis pandemic presents a massive challenge to global tuberculosis control, while in sub Saharan Africa TB and HIV are colluding to unfold "a tragedy of unprecedented proportions" (Nelson Mandela, XIII International AIDS Conference, Durban, 14/07/2000).

Efforts to contain the TB pandemic are further threatened by the emergence of multi-drug resistant TB (MDR-TB). The problem of *Mycobacterium tuberculosis* simultaneously resistant to rifampicin and isoniazid (the most potent antituberculosis agents currently available) has arisen where tuberculosis treatment regimens have been ill conceived or poorly applied. An alarming example is provided by the steep climb in resistance rates in the former USSR from the mid 1990s, following the breakdown of health care systems. The agents used to treat MDR-TB are relatively ineffective, toxic and costly.

Figure 2: Interventions to "interrupt" the sequence of events by which HIV fuels the TB epidemic: access to health care facilities providing intensified efforts to find and cure cases of TB, tuberculosis preventive treatment and interventions against HIV form the basis of the World Health Organization's proposed guidelines for national implementation strategies. The uninterrupted arrows show how HIV infection feeds the tuberculosis epidemic: HIV increases the risk of M. tuberculosis infection following exposure; HIV promotes the progression of tuberculosis infection to disease; the increased incidence of tuberculosis in people with HIV infection poses an increased risk of tuberculosis transmission in the community; and HIV increases the risk of recurrent tuberculosis. (adapted from: World Health Organization. Strategic framework to decrease the burden of TB/HIV. WHO Geneva, Switzerland 2002: WHO/CDS/TB/2002.296.)



Tuberculosis control

Fewer than 5% of patients with drug sensitive pulmonary tuberculosis who are fully treated with rifampicin-based SCC should fail treatment or suffer disease relapse[19,20]. The global TB control initiative of the World Health Organization (WHO) aims by 2005 to detect 70% of smear positive patients and treat successfully 85% of these cases

through support of DOTS (directly observed treatment, short course) programmes implementing SCC. Despite recent increases in both TB incidence and prevalence, cure rates have steadily improved in the Western Cape region, from 65% in 1997 to 73% in 2002[21]. However, major challenges obstructing successful treatment lie in adequate treatment delivery and regular adherence to the cumbersome treatment regimens.

Much publicity has been given to the hunt spearheaded by the Global Alliance for TB Drug Development, for a new drug that will reduce treatment duration and dose frequency, be safe, and effective against MDR tuberculosis. The aim of the Global Alliance is to have a new drug in production by 2010[22], and it will only be effective if applied in the context of a broad strategy to prevent, detect and treat the disease.

A strategy to combat TB must include effective interventions to curb HIV infection and AIDS, and the spread of MDR TB. Substantially increased aid flows are required for poverty alleviation programmes, and to support interventions in the home, the community and health care facilities. Access to health care facilities providing intensified efforts to find and cure cases of TB, tuberculosis preventive treatment and interventions against HIV form the basis of the World Health Organization's proposed guidelines for national implementation strategies[23] (figure 2). Prevention of MDR TB through reliable drug supplies and good adherence to effective multiple drug containing regimens is an important priority of TB control programmes.

Many more will fall victim to tuberculosis before the pandemic is brought under control.

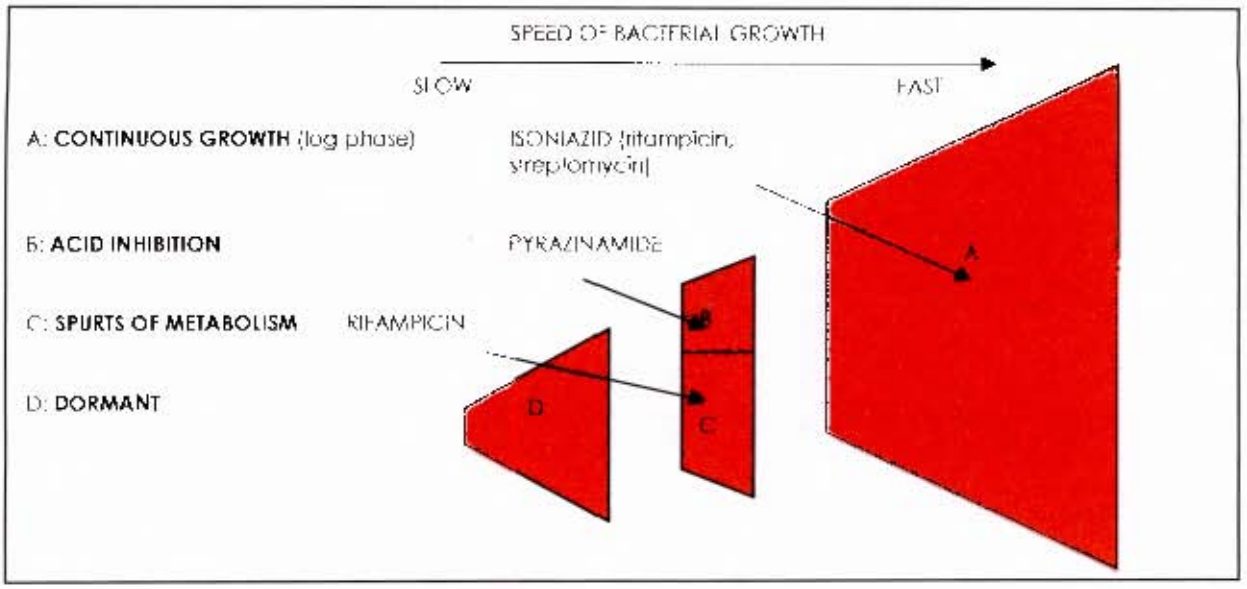
Antituberculosis treatment

Antituberculosis chemotherapy was introduced in the 1950s with streptomycin. It was rapidly recognized that monotherapy allowed the early development of resistance. Two- and three-drug combinations were therefore introduced to prevent the emergence of resistance [9].

In the 1970s it was recognized that treatment regimens could be substantially shortened by the use of rifampicin and pyrazinamide in 'short-course' treatment regimens. These drugs are able to kill those slowly dividing, metabolically sluggish, persisting organisms, thus sterilizing tuberculous lesions and preventing disease recurrence in the months following treatment. It is the sterilizing action of the regimens

that determines the requisite duration of drug therapy[23]. The 'special populations' hypothesis[24] (figure 3) explains how a combination of first line antituberculosis drugs acts synergistically to eradicate organisms in different metabolic phases.

Figure 3: Special populations hypothesis for the action of antibacterial drugs on different parts of the bacterial population in human lesions (adapted from: DA Mitchison. The Garrod lecture: Understanding the chemotherapy of tuberculosis - current problems. Journal of Antimicrobial Chemotherapy 1992; 29: 477-493)



Bactericidal activity against rapidly dividing (log phase) organisms is reflected by the rate of kill during the first 2 days of treatment measured by the decline in colony forming units in the sputum i.e. the early bactericidal activity (EBA). Sterilizing activity corresponds to the time to kill the persisting organisms and is measured by the prevention of disease relapse after the end of treatment (or by the surrogate marker, sputum culture 2 months after commencing therapy).

A standardised approach is used in most treatment programmes to facilitate large-scale treatment programmes in primary health care settings. In South Africa standard rifampicin based regimens are used as advocated by the WHO (figure 4). The initial intensive phase comprises rifampicin, isoniazid, pyrazinamide and ethambutol (with the addition of streptomycin in patients who have previously received tuberculosis treatment for more than 4 weeks). During the intensive phase, the actively dividing (log phase) bacilli should be eliminated and the number of organisms harboured by the patient reduced to a few persisting organisms thereby minimizing the possibility of drug resistance developing during the continuation phase. Approximately 90% of patients have no viable *M. tuberculosis* in sputum cultures after 2 months of intensive phase treatment[19]. The continuation phase aims to eradicate those persisting

organisms and comprises rifampicin and isoniazid in combination (with ethambutol in addition for retreatment patients).

Figure 4: Standard treatment regimens advocated by the South African Tuberculosis Control Programme for adults with drug susceptible tuberculosis (from: The South African Tuberculosis Control Programme Practical Guidelines 2000)

REGIMEN 1 – NEW ADULTS
New smear positive patients (and other serious pulmonary and extrapulmonary tuberculosis cases) who have never been treated before or have previously been treated for less than 4 weeks.

Intensive Phase – 2 months	< 50 kg	≥ 50 kg	Continuation Phase – 4 months	< 50 kg	≥ 50 kg
Rifampicin	480 mg	600 mg	Rifampicin	450 mg	600 mg
Isoniazid	240 mg	300 mg	Isoniazid	300 mg	300 mg
Pyrazinamide	1200 mg	1500 mg	-		
Ethambutol	800 mg	1000 mg	-		

All drugs given daily, 5 days a week; FDCs recommended

If conditions do not allow for giving treatment 5 times a week in the continuation phase, treatment can be given 3 times a week	Alternative Continuation Phase – 4 months	< 50 kg	≥ 50 kg
	Rifampicin	450 mg	600 mg
	Isoniazid	400 mg	600 mg

REGIMEN 2 – RETREATMENT OF ADULTS
Smear positive retreatment patients (relapse, failure, or interruption of treatment)

Intensive Phase – 3 months	< 50 kg	≥ 50 kg	Continuation Phase – 5 months	< 50 kg	≥ 50 kg
Rifampicin	480 mg	600 mg	Rifampicin	450 mg	600 mg
Isoniazid	240 mg	300 mg	Isoniazid	300 mg	300 mg
Pyrazinamide	1200 mg	1500 mg	Ethambutol	800 mg	1200 mg
Ethambutol	800 mg	1000 mg			
Streptomycin (2 months only)	750 mg	1000 mg			

All drugs are given daily, 5 days a week; FDCs recommended

If conditions do not allow for giving treatment 5 times a week in the continuation phase, treatment can be given 3 times a week.	Alternative Continuation Phase – 5 months	< 50 kg	≥ 50 kg
	Rifampicin	450 mg	600 mg
	Isoniazid	400 mg	600 mg
	Ethambutol	800 mg	1600 mg

Streptomycin dose should be reduced to 750 mg in patients older than 40 years, and not be given to those over 65 years or during pregnancy. Dosage adjustment is required in patients with impaired renal function.

Rifampicin-based, short-course regimens and directly observed treatment

The widely recommended DOTS (the brand name of the internationally recommended tuberculosis control strategy: Directly Observed Treatment, Short-course) concentrates on case-finding and cure. Approximately 69% of the world's TB patients live in areas now covered by DOTS programmes[2]; yet TB control programme results are still severely hindered by the lack of better and affordable diagnostics and more effective medicines[22]. The currently recommended antituberculosis treatment comprises short course chemotherapeutic regimens (SCC) of 6 to 8 months, which contain rifampicin for at least the first 2 months (during the intensive phase). The

preferred regimens contain rifampicin throughout; this allows the treatment course to be shortened to 6 months for those on TB treatment for the first time. However, concerns of increased resistance to rifampicin have led the World Health Organization (WHO) to strongly recommend that all rifampicin doses should be strictly supervised. Non-rifampicin-containing continuation phases have therefore been used widely in countries unable to support directly observed treatment for the full duration. Recent evidence suggesting worse outcomes for HIV-infected patients if the continuation phase of treatment does not contain rifampicin[25,26]; increased access to drugs through the Global Drug Facility; and increased support for DOTS programmes by the Global Fund to Fight AIDS, Tuberculosis and Malaria may lead to more programmes using rifampicin throughout the treatment course.

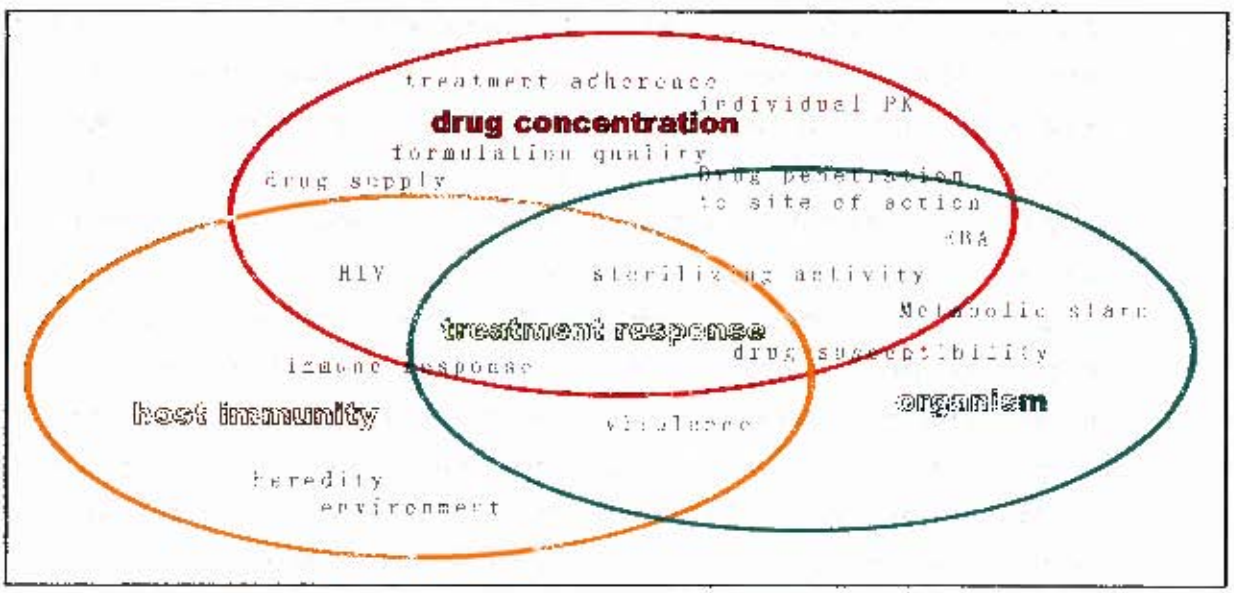
The use of fixed-dose combinations (FDCs) of antituberculosis drugs is encouraged. They hold several advantages including simplified procurement and prescribing practices and a reduced risk of acquired drug resistance. Because the drugs are administered in combination, resistant mutants are less likely to be selected when FDCs are used in the setting of erratic drug taking or treatment interruption[25]. While the use of FDCs is recommended by the WHO and the IUATLD (International Union Against Tuberculosis and Lung Disease), reports of inferior bioavailability of rifampicin in several FDCs[29-32] have lead to a joint statements by the organisations that only those FDCs with proven rifampicin bioavailability should be used[33,34].

Mechanisms to ensure a reliable supply of drugs known to be of good quality are an important aspect of implementation strategies and are supported by the recently established Global Drug Facility (GDF)[35]. Of special concern is the bioavailability of rifampicin; the quality of the raw material (the particle size and crystalline form)[36], the nature of the excipients and exposure to heat and humidity during the manufacturing process are reputed to be important factors. Recent reports of accelerated degradation of rifampicin in FDCs under conditions of high ambient temperatures (40°C), humidity (75%RH) and light, imply that storage conditions may be very important[37]. It is hypothesized that an interaction between isoniazid and rifampicin, which may be augmented by the presence of ethambutol under humid conditions, is responsible[37]. Furthermore, the drug is relatively insoluble in water, is subject to enterohepatic circulation[38,39] and dissolution testing does not correlate well with bioequivalence[40]. *In vivo* testing of the bioavailability of rifampicin-containing products in human volunteers is therefore a generally accepted requirement prior to registration[41].

The treatment success gap

Initial trials of rifampicin-based SCC regimens had success rates of more than 95%, but a small proportion of patients failed treatment or had relapse of disease in spite of the controlled study environment[19,55]. Conceivably, sub-optimal systemic drug exposure could play a role in those patients who have a poor treatment response even under the study conditions of excellent and assured drug quality and delivery, fully drug sensitive organisms, with clinical disease confined to the lung, and limited or no co-morbid illness. If low drug concentrations in some individuals are contributory under optimal conditions of drug delivery, they might be equally or even more important in widely implemented treatment control programmes. Low drug levels may be more critical in the context of incomplete drug delivery, sub optimal drug quality, an array of disease presentations (the site and metabolic state of the organism being relevant), severely ill patients with co-morbid disease and malnutrition who may be vulnerable to altered drug pharmacokinetics, and compromised immunity. In addition, the possibility should be entertained of sub-optimal quality drugs being particularly poorly absorbed in patients with more severe disease or malnutrition. It is likely that treatment failure or tuberculosis recurrence results from a complex combination of factors that varies from patient to patient[41]. The relative importance of the interacting determinants of outcome is poorly understood. (figure 5)

Figure 5: Interacting and poorly understood factors determine therapeutic outcome.



Non-adherence to treatment regimens is the most important risk factor known to be associated with poor treatment outcome. However, certain patients are more prone to disease relapse; extensive pulmonary cavitation, extensive radiographic changes

on the pretreatment chest X-ray, male sex, and advanced age are putative risk factors[3,42,45]. Smoking, diabetes, low body mass, and a previous history of treatment for tuberculosis have also been associated with worse treatment outcomes[42,47,46]. HIV-infected patients are more likely to relapse after rifampicin-sparing chemotherapy (6 month regimens with twice-weekly rifampicin in the continuation phase[48], 8-month regimens with only 2 months of rifampicin[25], 12 month regimens without rifampicin[26]); and the presence of extrapulmonary disease in addition to pulmonary tuberculosis and drug abuse are independent risk factors in this group[49,50]. Furthermore, HIV-infected patients are at a higher risk of relapsing with rifamycin resistant *M. tuberculosis* than HIV negative patients when highly intermittent dosing regimens are used[51]. These patients may require doses at the top end of the dose-response curve or more frequent doses for optimal treatment outcome.

The inherent susceptibility of *M. tuberculosis* to the antituberculosis agents may vary between different isolates (even amongst those organisms categorized as drug sensitive). A more refractory metabolic state of *Mycobacterium tuberculosis* may be responsible for the increased drug tolerance observed in isolates from patients with prolonged bacterial persistence or disease relapse[171]. Those patients infected with strains having higher MICs can be expected to require higher drug doses to achieve therapeutic response, and this may be important in patients achieving drug concentrations below the normal ranges.

The quality of drug products and delivery of adequate drug doses is emphasized. However, as is evident from the studies summarized in the following section, the relationship between drug plasma concentrations and treatment outcome in tuberculosis patients has not been defined, and factors associated with low drug levels in patients have not been adequately described.

Drug concentrations in tuberculosis patients

Several isolated reports of low antituberculosis drug concentrations in patients have emerged in the literature since the early 1990's.

The case of an HIV-infected patient with a malabsorptive disorder and disseminated tuberculosis was described in a letter published in The New England Journal of Medicine in 1992[52]. The concentrations of rifampicin, isoniazid and pyrazinamide at 2 and 6 hours after drug ingestion were found to be low when compared to the "normal range" for the peak concentration. (Table 1; Case i).

Table 1: Initial reports of low antituberculosis drug concentrations

Drug:	Isoniazid	Rifampicin			Pyrazinamide		Ethambutol
Recommended 2 hour range (mg/l)[52,53]	3.0 – 5.0	8.0 – 24.0			20 – 60		2 – 6
CASE I: (Berning et al.)							
Dose:	300mg	600 mg	900 mg	1200 mg	1500 mg	2000 mg	Not reported
Plasma concentration (mg/l):							
After 2 hours:		1.7	7.1	13	41	50	Not reported
After 6 hours:	0.13	2.6	3.2	9.1	22	42	Not reported
CASE II: (Patel et al.)							
Dose:	300 mg	600 mg			800 mg		Not reported
Plasma concentration (mg/l): After 2 hours:	0.8	0			24		0.4
CASE III: (Patel et al.)							
Dose on 1st occasion:	300 mg	600 mg			1500 mg		1500 mg
Plasma concentration (mg/l): After 2 hours:	0.92	0			35		0.86
Dose on 2nd occasion:	400 mg	600 mg					
Plasma concentration (mg/l): After 0.5, 1, 1.5 hours	ND	ND					
After 2 hours:	0.43	0.42					
After 4 hours:	ND	3.01					
After 6 hours:	ND	1.37					
After 8 hours:	ND	1.07					
After 12 hours:	ND	0.58					

ND: not detectable

Subsequently, another report from the United States described low plasma concentrations of isoniazid, rifampicin, ethambutol, ciprofloxacin and, less dramatically, pyrazinamide 2 hours after drug ingestion in 2 HIV-infected patients[55] (Table 1: Cases II and III). The levels of isoniazid, rifampicin and pyrazinamide are shown in Table 1. One patient had evidence of malabsorption (Case III); the other did not (Case II). Both acquired drug resistance (isolated rifampicin resistance in one patient and resistance to both rifampicin and isoniazid in the other) during treatment.

Cases of low drug concentrations in severely ill TB patients without HIV-infection were also described. A man with extensive pulmonary tuberculosis failed to respond to oral therapy. Rifampicin concentrations measured one and three hours after dosing were 0.6 and 0.5 mg/l, respectively. Clinical improvement was noted only after the rifampicin and isoniazid doses were increased and given intravenously[54]. In another case report, low rifampicin concentrations were implicated in the death of a woman with drug sensitive pulmonary tuberculosis and no known immunosuppression. The concentration of rifampicin at 2 hours after ingestion was 2.76 mg/l[56].

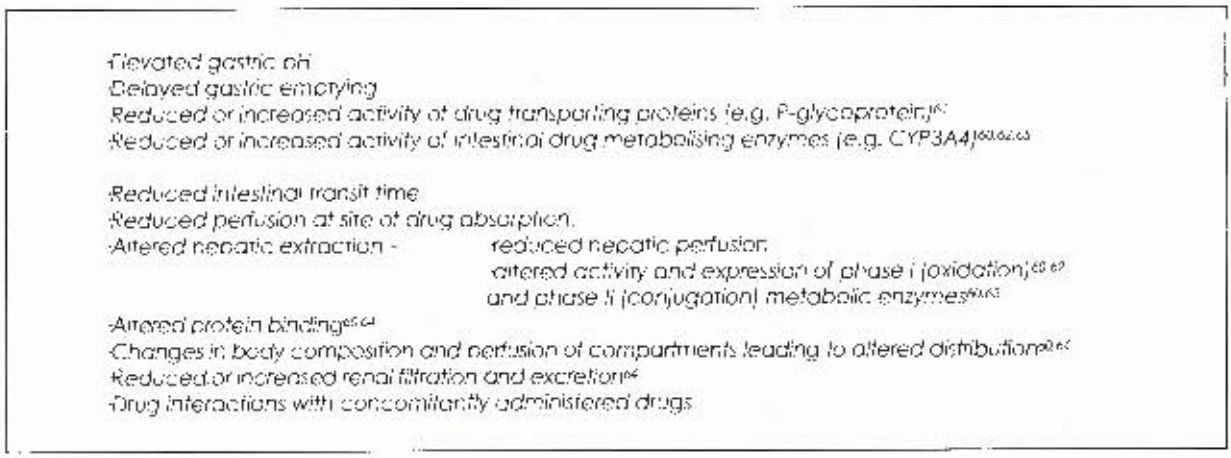
In the absence of target drug concentration ranges for patients, the plasma concentrations measured were compared to published recommended drug concentration ranges derived largely from healthy volunteer studies[56,57]. These ranges are not appropriate for patients established on therapy (see discussion below). For most of these patient cases, screening for low drug concentrations used a single 2 hour blood sample; the possibility of delayed absorption (which may occur more frequently in patients than in healthy volunteers, eg. through delayed gastric emptying), was not accounted for. In addition to the possibly spurious assumption of low drug bioavailability in some cases, other factors may have contributed to the poor response to treatment e.g. low drug doses.

Subsequently the findings of several studies describing antituberculosis drug concentrations in patients have been published.

Peloquin et al. studied the serum concentrations of antituberculosis drugs in a series of 26 patients with AIDS in the United States and Puerto Rico[58]. they found that most patients had low 2-hour levels of rifampicin (about 79% of patients), ethambutol (about 83% of patients) and isoniazid (about 60% of patients) in comparison to the proposed normal ranges. Two patients had very low (< 30% of the lower limit proposed 2 hour concentration) concentrations of rifampicin and isoniazid. In spite of the prevalence of low concentrations of the two most active antituberculosis agents it was noted that patients generally seemed to have a good response to treatment.

Unfortunately, no control subjects without AIDS were studied. Instead, the blood concentrations measured were compared to the recommended 2-hour concentrations derived from values in the literature and pharmacokinetic studies[58,59]. The proposed ranges largely reflect the concentrations in healthy volunteers and are not suitable for patients established on therapy. In particular, substantially lower levels of rifampicin are expected as autoinduction occurs with repeated doses[29,59]. Furthermore, disease and malnutrition may alter absorption, distribution, metabolism and elimination of drugs by several mechanisms (although limited evidence is available in this poorly defined field; figure 6)

Figure 6: Disease may alter the pharmacokinetics of drugs by several mechanisms.



For most patients, a single blood sample taken at 2 hours after drug ingestion was used to derive the drug concentrations. A single sample cannot represent the peak concentration for a number of different drugs; for example, the maximal concentration of isoniazid in most patients is achieved earlier than 2 hours after drug administration, and that for ethambutol later[56,57,66,68]. Also, scant sampling does not accurately reflect the peak drug concentration during a dosing interval as the time to peak drug concentration varies widely between patients. Indeed, Peloquin et al. found that the rifampicin concentration at 6 hours after drug administration was higher than that at 2 hours in 3 of the 4 patients sampled at both times.

The implication of several of the emerging reports that low drug concentrations are linked to HIV-infection (or AIDS), is supported by the findings of Sahai et al[69]. Healthy volunteers and HIV-infected persons without tuberculosis were given antituberculosis drugs for 4 days. Serial blood samples were then taken over 24 hours to generate concentration-time curves for rifampicin, isoniazid and pyrazinamide. The mean peak concentration (Cmax) and the area under the concentration-time curve (AUC) over 24 hours of rifampicin were decreased by 41% and 32%, respectively, in HIV-infected persons. The extent of the decrease was related to the severity of HIV-infection. In those with asymptomatic HIV-infection and CD4+ cell count above 300 cells/mm^3, the mean Cmax and AUC values were reduced by 36% and 29%, respectively, compared to those of healthy volunteers. In those with symptomatic HIV-infection, CD4+ counts below 200 and cells/mm^3 and without diarrhoea the reductions were 43% and 31%, respectively; and in those with AIDS and chronic diarrhoea the most marked reductions were seen in pharmacokinetic measures, with values of 44% and 35%, respectively. Pyrazinamide showed a similar trend, although the mean peak concentration and AUC values were reduced in HIV-infected subjects to a lesser

extent than for rifampicin. The maximal concentration of isoniazid was significantly delayed and reduced in those patients with AIDS and chronic diarrhoea, but was not affected in AIDS patients without diarrhoea or those with asymptomatic HIV-infection. The altered pharmacokinetics with HIV-infection was attributed to malabsorption of the drugs. However, changes in D-xylose absorption accounted for only 31% and 17% of the variability of rifampicin AUC and pyrazinamide AUC, respectively. As the study did not include TB patients the pharmacokinetic impact of tuberculosis or co-infection with tuberculosis and HIV was not evaluated.

Conversely, studies in tuberculosis patients from Kenya and South Africa failed to show significantly lower drug concentrations in patients with TB/HIV co-infection, compared to tuberculosis patients without HIV-infection[70,71]. Furthermore, a study in Thai tuberculosis patients with AIDS demonstrated good peak concentrations of rifampicin (mean Cmax 9.81 mg/l + 4.41 mg/l), although no control group data (from tuberculosis patients without AIDS) were reported[72].

In the Kenyan study, neither HIV-infection nor diarrhoea was associated with the variability in the pharmacokinetic measures Cmax, AUC or terminal half-life of rifampicin, isoniazid or pyrazinamide. In this study 14 HIV-infected and 15 non-HIV-infected patients with tuberculosis were admitted to hospital and commenced on standardized doses of rifampicin (600 mg /daily dose if ≥ 45 kg; 450 mg if < 45 kg), isoniazid (300 mg/day), pyrazinamide (1.5 g/day) and ethambutol (800 mg/day). Pharmacokinetic profiles of rifampicin, isoniazid and pyrazinamide were determined after 2 weeks of treatment from blood samples drawn just prior to drug administration, and at 0.5, 1, 1.5, 2, 4, 6, 8 and 12 hours after drug ingestion. As shown in table 2, the levels of rifampicin and isoniazid were markedly reduced in comparison to the recommended ranges in the literature and highly variable, whilst those of pyrazinamide were generally within the recommended range and less variable. The non-normalized Cmax values were below the recommended ranges in the literature in 90% and 89% of patients for rifampicin and isoniazid, respectively. The authors suggest that malnutrition or wasting may have contributed to malabsorption in the patients studied. Most of the patients studied were cachectic; the mean body mass index was 17.0±2.1 kg/m^2. The consequence of such low drug levels was not assessed in this study.

The study in Cape Town in 13 tuberculosis patients with AIDS and 14 patients without HIV-infection also demonstrated that HIV infection was not associated with lower concentrations of rifampicin, isoniazid or pyrazinamide (table 2). The levels reported

were higher than those in the Kenyan study and the body mass index was, on average, higher – but differences in the dosing regimens and the sampling schedules may be partly responsible for the higher Cmax values reported (in the study in Cape Town, samples were drawn at 20 minute intervals between 2 and 4 hours). also, the pharmacokinetic measures were not normalized to a 70 kg body weight.

Table 2: Pharmacokinetic measures of rifampicin, isoniazid and pyrazinamide in TB patients established on treatment regimens (unless otherwise stated*). Cross comparison should be with caution as different patient selection criteria, dosing schedules and sampling schedules were used, and different analytical techniques for were used for drug concentration determination.

STUDY (sampling [illegible])	**Rifampicin**			**Isoniazid**			**Pyrazinamide**		
Normal range for Cmax[1,2,3]	8-24 (600 mg dose)			3-5 (300 mg dose)			30-50[1] (20-25 mg/kg dose)		
Acocella et al.[29] Dose: 9.7-12.9mg/kg	Cmax mg/l	AUC (0-12h) mg.h.l^{-1}	$T_{1/2}$ h	Cmax mg/l	AUC(0-12h) mg.h.l^{-1}	$T_{1/2}$ h	Cmax mg/l	AUC (0-12h) mg.h.l^{-1}	$T_{1/2}$ h
[illegible]	(mean ± sem)			(mean ± sem)			(mean ± sem)		
*After 1st dose (n=13)	9.9 ± 0.9	57.9 ± 6.8	2.6 ± 0.3	8.0 ± 0.6	31.7 ± 3.4	2.5 ± 0.2	37.8 ± 1.7	262 ± 17	9.9 ± 1.1
After 60 days of treatment (n=13)	9.5 ± 0.9	44.3 ± 4.2	2.0 ± 0.4	7.6 ± 0.5	32.3 ± 3.0	2.5 ± 0.1	41.7 ± 2.4	319 ± 20	7.7±0.5
Zent et al[73] (n=27) [illegible]	Cmax mg/l	AUC(0-8h) mg.h.l^{-1} (mean ± sd)	Tmax h	Cmax mg/l	AUC(0-8h) mg.h.l^{-1} (mean ± sd)	Tmax h	Cmax mg/l	AUC(0-8h) mg.h.l^{-1} (mean ± sd)	Tmax h
PK without food	7.5±4.1	25.8±15.9	2.5±0.7	4.4±2.0	22.1±10.1	1.7±0.7	58±17	314±117	7±0
Chaudri et al.[47] [illegible]	Cmax mg/l	AUC mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ h	Cmax mg/l	AUC mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ h	Cmax mg/l	AUC mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ h
All patients (n=29) non-normalised data	5.2±2.67			1.8±0.88					
NOTE applying to 4 rows below:	Data normalized to 600 mg dose and 70 kg body weight			Data normalized to 300 mg dose and 70 kg body weight			Data normalized to 1.5 g dose and 70 kg body weight		
HIV/TB (n=14)	4.1±2.0	23.1±12.9	2.8±1.2	1.4±0.8	7.9±7.3	3.2±1.6	32.1±8.8	350±111	7.4±1.6
Not HIV infected (n=15)	4.3±2.4	19.6±10.6	2.0±0.7	1.1±0.4	6.3±3.5	3.3±0.9	33.1±8.2	392±142	6.9±2.1
diarrhoea (n=11)	4.2±2.1	24.0±13.9	2.8±1.4	1.3±0.5	8.8±6.3	3.5±1.4	33.9±9.0	427±148	7.8±1.9
No diarrhoea (n=18)	4.1±2.3	19.3±9.9	2.1±0.6	1.2±0.7	5.7±4.8	2.9±1.7	31.7±8.1	326±123	6.7±1.8
Talla et al.[71] [illegible]	Cmax mg/l	AUC mg.h.l^{-1} Median (range)	Tmax h	Cmax mg/l	AUC mg.h.l^{-1} median (range)	Tmax h	Cmax mg/l	AUC mg.h.l^{-1} median (range)	Tmax h
HIV/TB (n=13)	12.5 (6.1-19.5)	60.1 (30.5-88.4)	2.7 (1-4)	6.5 (3.3-10.8)	22.5 (10.5-50.4)	1.3 (0.5-1.8)	56 (47-72)	378 (247-601)	7.3(0.3 -1.7)
Not HIV infected (n=14)	7.4 (1.4-31.9)	27.8 (2.2-102.8)	2 (1.3-3.25)	6.1 (3.6-19.7)	17.7 (5.2-52.9)	0.8 (0.3-2)	57 (45-70)	385 (232-499)	7.3 (0.3-9)
Kimerling et al.[74] (n=14)	2-hr level (mg/l) after 600 mg dose (mean ± sd) 5.37+4.14			2 hour level (mg/l): 900 mg dose (mean ± sd) 7.37+6.27					
Van Crevel et al.[75]	2-hr level (mg/l) after 450 mg dose (median)								
Males (n=43)	2.57								
Females (n=19)	4.62								
Polasa et al.[64] (n=10) [illegible]	Cmax mg/l	AUC0-∞ mg.h.l^{-1} (mean + sem)	$T_{1/2}$ hr						
	4.13 ± 0.53	24.11 ± 2.28	4.76 ± 0.45						
Mehta et al.[76] 'slow responders'**	2-hr concentration (mg/l) (median (range))								
600 mg dose (n=6)	1.25 (<1.0 - 5.9)			**The same 6 patients received 600 and 900 mg doses					
900 mg dose (n=6)	11.85 (1.0-15.2)								

Table 2 continued

Own data; DP Marias Hospital *(n=65)*	Cmax mg/l	AUC_{0-4} mg.h.l^{-1}	$T_{1/2}$ hr	Cmax mg/l	AUC_{0-4} mg.h.l^{-1}	$T_{1/2}$ hr	Cmax mg/l	AUC_{0-24} mg.h.l^{-1}	$T_{1/2}$ hr
mean ± sd	9.4±3.5	21.7±12.3	1.7±0.4	7.4±4.0	30.8±20.6	2.8±0.9	40.0±17.2	379±167.2	6.9±1.1
	2-hr concentration: 7.0±3.2			2-hr concentration: 4.9±2.6			2-hr concentration: 34.8±15.5		
Median (range)	9.0	31.7	1.6	6.4	25.0	2.5	39.4(96.3	371	6.8(4.8-
	(1.6-15.1)	(4.1-61.0)	(0.9-3.5)	(1.9-16.9)	(5.9-95.4)	(1.2-5.1)	-65.1)	(259-627)	10.4)
(.25, .5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24)	Median 2-hr concentration: 7.00(0-14.04)			Median 2-hr concentration: 4.22(1.35-11.01)			Median 2-hr concentration: 34.75(24.33-50.95)		
Israili et al.[77] 600 mg dose (1, 2, 3, 4, 6, 8, 12)	Cmax mg/l	AUC_{0-x} mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ hr	Cmax mg/l	AUC_{0-x} mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ hr			
*After 1st dose (rifamycin: n=16; isoniazid: n=14)	9.8 ± 4.4	47.0 ± 34.5	2.9 ± 0.9	4.5 ± 2.0	22.9 ± 9.9	3.1 ± 1.1			
Rifampicin: after 15th (6th dose (n=12) Isoniazid: after 7th – 15th dose (n=12)	6.5 ± 3.5	27.0 ± 4.8	1.7 ± 0.5	3.9 ± 1.7	18.9 ± 9.9	3.0 ± 0.9			
Lopez-Cortez et al.[78] *(10.7 ± 1.0 mg/kg)* (1, 2, 3, 4, 6, 8, 12)	Cmax mg/l	AUC_{0-12} mg.h.l^{-1} (Mean ± sd)	$T_{1/2}$ hr						
*7 days after treatment initiation (n=8)	5.9 ± 4.4	25.9 ± 18.2	1.3 ± 0.8						

[a] The ranges for normal peak concentrations in the literature are generally similar to the 2-hour range proposed by Peloquin[54]

[b] The 2-hour range proposed by Peloquin is 20-60 mg/l

In an observational study[74], investigating the concentrations of rifampicin and isoniazid at 2 hours after drug ingestion in non-HIV-infected adults with pulmonary tuberculosis who were not responding well to treatment, the drug formulation and alcohol use were associated with drug concentrations. Rifampicin concentrations were found to be higher ($p < 0.02$) and isoniazid levels lower ($p < 0.04$) in patients given a fixed dose combination tablet of the two drugs compared to the concentrations measured in patients ingesting single drug products. Alcohol use was associated with higher rifampicin concentrations ($p < 0.01$).

Van Crevel et al.[75] found that females had higher drug concentrations than males (table 2). However, the most important determinant of the 2-hour rifampicin concentration was the formulation. Those patients taking tablets supplied by one particular manufacturer had significantly lower concentrations, in spite of adequate rifampicin content of the tablet.

The recently published findings of a small study investigating rifampicin concentrations in the alveolar cells and epithelial lining fluid (obtained by bronchoalveolar lavage), and plasma of men and women with and without AIDS, but without tuberculosis, failed to show an effect of AIDS or sex on plasma levels at 2 or 4 hours after drug administration. However a there were trends to higher alveolar cell rifampicin levels (obtained at 4 hours after drug dosing) in women than in men, and in smoking women than in non-smoking women.[79]

In a study investigating the impact of undernutrition on the pharmacokinetics of rifampicin, Polasa et al.[64] found that 8 subjects with mean body mass index (BMI) of 14.8 kg/m^2 had significantly lower drug concentrations than 10 healthy subjects with BMIs above 18 kg/m^2 (mean BMI 21 kg/m^2) after a single 10 mg/kg dose: the mean Cmax(± sem) values were 5.61 (+ 0.50) and 11.09 (± 1.27) mg/l, respectively, and the AUCs 42.24 (± 4.22) mg.hr/l and 59.12 (± 4.91) mg.hr/l, respectively. The authors cite malabsorption, changes in distribution and increased renal clearance as probable reasons for the differences observed. Ten undernourished tuberculosis patients with a mean BMI of 16.7 kg/m^2, established on a rifampicin containing treatment regimen, had further reductions in the bioavailability of the drug (mean Cmax (± sem) of 4.13 (± 0.53) mg/l, and AUC 24.11 (± 3.28) mg.hr/l). Unfortunately the study lacked well nourished tuberculosis patient controls.

Reduced bioavailability of isoniazid and rifampicin is observed in patients taking food concomitantly.[75,80] Zent et al. found that ingestion of a high carbohydrate meal with antituberculosis medication delayed rifampicin absorption, and decreased Cmax and AUC values for isoniazid by approximately 20% in a group of 27 patients in the Western Cape. Concomitant ingestion of a meal high in lipid caused a smaller reduction in isoniazid levels. Pyrazinamide concentrations were unaffected. Most of the reported patient studies have ensured drug administration on an empty stomach, thus eliminating this source of variability.

The results of 2 reports[74,76] suggest that a high proportion of patients responding poorly to first line therapy have drug concentrations below the target ranges in the literature. However, the drug levels in patients responding adequately to treatment were not measured in either study. Therefore, it cannot be concluded that a high proportion of patients with low drug levels have inferior treatment responses.

Kimerling et al.[74] measured isoniazid and rifampicin concentrations in non-HIV infected patients with a slow clinical response (failure to convert the sputum culture within 12 weeks), treatment failure or disease relapse within 13 months of being cured or acquired drug resistance while receiving directly observed therapy. Fourteen of the 22 (64%) patients receiving first-line treatment, had 2-hour rifampicin concentrations less than 8 mg/l (the lower limit of the published target range) and 6 of the 8 patients (75%) receiving a 300 mg dose of isoniazid had 2-hour concentrations less than 3 mg/l (the lower limit of the 2-hour reference range for isoniazid[56]; table 2).

Mehta et al.[74] identified 6 of 124 patients treated for pulmonary tuberculosis for the first time who were responding poorly after 3 months on standard treatment (positive sputum smear or culture, or poor clinical or radiographic response). All six patients were found to have low concentrations of rifampicin (less than 8 mg/l) at 1.5 and 2.5 hours after drug ingestion under fasting conditions (table 2). The dose of rifampicin was increased from 600 mg to 900 mg and then to 1200 mg in one patient. Disproportionately increased rifampicin levels were measured at the higher doses. Improved responses to treatment were observed following the dose increments. Too few patients were examined to draw conclusions about factors associated with the low levels, but only one of the patients was infected with HIV and half of the patients were alcoholics.

Without control subjects it cannot be concluded whether low drug levels are significantly associated with the inferior response to treatment of the patients in these studies. However, circumstantial evidence that low drug concentrations are responsible for poor treatment response in some patients is provided by reports of several cases responding well to increased doses of antituberculosis drugs, after low concentrations had been detected on initial standard doses.[52,54,74]

In contrast, Narita et al. imply that drug concentrations are not important predictors of tuberculosis recurrence[81]. They measured rifampicin and isoniazid concentrations in approximately half of the 188 patients in their study designed to determine the risk of recurrence associated with low serum concentrations of the 2 drugs, HIV infection, age, sex, silica dust exposure, injection drug use, CD4 cell count less than 100 cells/ml and HIV RNA load greater than 150 000 copies/ml. The findings in 25 (13%) patients with tuberculosis recurrence were compared to those being treated for the first time. A multivariate logistic regression analysis was used to assess the risk of recurrent tuberculosis associated with the above-mentioned covariate factors, and none were found to be significant determinants. The results are inconclusive for several reasons. The assumption was made that the measurements of covariate factors measured in patients with recurrent disease were representative of these factors at the previous treatment period, in spite of no indication in the report of the time to relapse from the previous episode of tuberculosis. The assumption holds for fixed factors such as sex, and may hold for factors such as silica dust exposure, injection drug use and HIV infection, depending on the timing the previous episode of tuberculosis and the onset of the risk factors. However, CD4 cell count, viral load and drug concentrations may well be quite different. Secondly, the scant sampling used to determine serum drug

concentrations (drug levels were measured at 2 and 6 hours after drug administration) might not accurately reflect drug exposure during that dosing interval as the time of the peak drug concentration varies. Thirdly, the serum drug concentration was dichotomised at the lower limit of their dose-specific expected ranges (< 3 mg/l versus ≥ 3 mg/l for patients receiving 300 mg doses of isoniazid daily; < 9 mg/l versus ≥ 9 mg/l for patients receiving 900 mg isoniazid twice weekly; and < 8 mg/l versus ≥ 8 mg/l for those receiving rifampicin 600 mg daily). As these expected ranges based largely on data from healthy volunteers are not appropriate for a patient population established on therapy, the impact of the lowest concentrations may have been missed in this analysis. Lastly, only 25 patients with recurrence of tuberculosis were included in the analysis. If drug concentrations were measured in approximately half of these (the exact number to undergo pharmacokinetic analysis was not reported), and the variability in drug concentrations is high as reported in other patient studies, the study may have been under-powered, as suggested by the wide 95% confidence interval limits.

The recently published findings of Weiner et al.[51] deserve attention, even though the retrospective nature of the pharmacokinetic evaluation has inherent weaknesses. The levels of rifamycin and isoniazid were evaluated in patients enrolled into Tuberculosis Trials Consortium/ United States Public Health Service Study 22, which compared the activity of once-weekly rifapentine plus isoniazid to twice-weekly rifampicin plus isoniazid during the continuation phase of antituberculosis treatment. In the primary evaluation, a multivariate analysis found that isoniazid levels were associated with an increased risk of poor treatment outcomes in those receiving the once-weekly regimen, but not in the group receiving drug doses twice a week. Rifampicin and rifapentine levels were not predictive of treatment outcome in this analysis. This is the first evidence that isoniazid concentrations may be an important determinant of treatment outcome, at least in highly intermittent dosing regimens. The association of acetylator status with treatment outcome strengthens the finding and is supported by previous studies linking rapid acetylator status and treatment failure on once-weekly regimens [9]. The data suggests that rifamycin and isoniazid levels are not important for treatment outcome in patients without HIV infection receiving twice-weekly regimens; however, this population specific finding should be interpreted with caution; the retrospective nature of the pharmacokinetic investigation might be particularly unreliable for predicting rifampicin levels and variation therein.

In summary, it is established that in many studies, patients have peak antituberculosis drug concentrations lower than the therapeutic ranges advocated in the literature, and that the low concentrations might in some circumstances be related to HIV-infection, alcohol use, undernutrition, male sex or drug formulation. The implications of these findings are not known. Appropriate ranges, for the drug concentrations or other pharmacokinetic measures, based on studies evaluating the effectiveness and toxicity of treatment have not been established.

In spite of poor characterisation of TB drug concentrations in patient populations, the unknown implications of low drug concentrations and the lack of validated methods for detecting sub-optimal drug exposure, the use of therapeutic drug monitoring has been advocated.[56,62]

Rifampicin: pharmacokinetic concerns

Systemic concentrations of rifampicin in the context of tuberculosis treatment are of particular concern for several reasons: the drug is known to have unpredictable bioavailability with highly variable plasma concentrations; it displays dose-dependent efficacy within the clinically relevant range; it is a drug of pivotal importance in contemporary short course antituberculosis chemotherapy; and it is associated with several important pharmacokinetic drug-drug interactions. In this thesis, greater attention will therefore be focussed on rifampicin than the other first-line antituberculosis agents.

The unique sterilising activity of rifampicin has made it possible for the duration of treatment regimens to be reduced substantially.[19,24,63,64] Together with isoniazid, it forms the backbone of modern short-course chemotherapy, and rifampicin is an invaluable part of treatment regimens where it can be afforded.

Rifampicin binds to the intracellular target DNA-dependent RNA polymerase, thereby inhibiting mRNA synthesis[65]. Mutations in the *rpoB* gene of *M. tuberculosis* confer a high level of resistance[65,66] and are found in most resistant isolates[67,68]. Approximately 1 in 10^8 organisms are resistant without drug selection pressure[69].

The minimum inhibitory concentration (MIC) of rifampicin for *M. tuberculosis* is about 0.25 mg/l in broth and 0.5 mg/l in agar[20]. The minimum bactericidal concentration (MBC) is about 1 mg/l in Tween containing medium, and it is estimated that the plasma rifampicin concentration required for the same bactericidal activity in

infected lung tissue is 10-fold to 15-fold that of the MBC[30]. Therefore, optimal antimycobacterial concentrations should theoretically approach 10 – 15 mg/l.

Reference ranges of 8 – 20 or 24 mg/l are widely quoted for the peak rifampicin concentration (Cmax)[56,57,93]. However, these ranges are based largely on healthy volunteer data, and, as discussed previously, are not appropriate for patients established on treatment. Indeed, most reported peak concentrations in patient populations established on treatment fall below this range (table 2), with free drug concentrations likely to be well below those thought to have optimal *in vitro* bactericidal activity in many patients. Furthermore the concentration of the unbound fraction at the site of action may be considerably lower than that in the plasma.

Concern regarding low rifampicin concentrations is supported by the dose dependency of the drug's activity. Studies in mice have demonstrated dose dependent efficacy over the range of 5 to 40 mg/kg[56]. A study in patients demonstrated significantly reduced early bactericidal activity of a 300 mg dose compared to 600 mg[54]. Patients receiving intravenous dosing with rifampicin (associated with higher systemic concentrations) achieved a negative sputum culture more rapidly than those who received oral rifampicin[94]. Furthermore, a large clinical study showed that doses less than 9 mg/kg are associated with worse treatment outcomes[95], and the combination of rifampicin (in the recommended doses) and isoniazid fails to prevent the emergence of isoniazid resistance in some patients.

While most patients are cured by standard treatment regimes provided there is good treatment delivery and adherence, it is possible that increased doses, at least in some patients, might be more effective, and it may also be possible to shorten the duration of treatment by such approaches[93,44].

The pharmacokinetic measure that corresponds best with antimycobacterial activity for rifampicin is not established. However, it is has been suggested that the Cmax : MIC ratio should be maximized, as rifampicin acts on intracellular target[96]. The importance of an adequate peak concentration rather than a sustained level is also supported by the long post antibiotic effect demonstrated *in vitro* and the efficacy of rifampicin based intermittent treatment regimens[63,90,96]. The AUC/MIC was found to be a better predictor of bacterial count reduction than Cmax/MIC or the time that rifampicin concentrations remained above the MIC, in a murine model[94].

Although some studies indicate almost complete absorption of rifampicin, data are insufficient[66]. More recent evidence suggests that this frequently is not the case, and that reduced bioavailability may, in part, be due to formulation characteristics[28,32,75,97,98]. Rifampicin is best absorbed on an empty stomach. Delayed and reduced absorption have been reported when it is taken with food[39,73]. Gastric pH may also influence absorption; acidification with histamine was observed to give levels 2 times higher than those observed after administration of the antacid Na bicarbonate[39]. Maximal concentrations are highly variable between studies and individuals, and are normally detected from 1 to 4 hours[56,66,82].

In plasma, 80 to 90% of rifampicin is protein bound[39,66]. The drug distributes well to lung, stomach wall, fat and breast milk; with concentrations greater than those in plasma 12 hours after drug administration,[39] but not approaching peak plasma levels[50]. Cerebrospinal fluid (CSF) penetration, estimated by the ratio of the AUC in the CSF to the AUC in plasma, is about 22% with uninflamed meninges[99]. The concentrations of rifampicin recovered in saliva and nasal secretions are less than 1 mg/l[20,100,101].

The major metabolite of rifampicin is the microbiologically active compound 25-desacetylrifampicin. It is relatively water-soluble and is eliminated along with unchanged rifampicin largely through biliary excretion and to a lesser extent through renal elimination. The excretory capacity of the liver is saturated at doses of 300 450mg causing the half-life of rifampicin, and correspondingly, the proportion of the drug eliminated in the urine to increase with larger doses[39,102]. With repeated doses, rifampicin is eliminated more rapidly and the half-life is reduced. The effect is more marked with 900 mg than 300 mg doses and is maximal at about 6 days[39,91]. The autoinduction is thought to result largely from induction of desacetylating hepatic esterase enzymes although induction of glucuronidation may also play a role[28,29,90,103]. The recovery of rifampicin and, to a greater extent, the recovery of desacetyl-rifampicin in the bile are increased after repeated dosing[39], suggesting that biliary excretion may also be induced. Rifampicin is a known P-glycoprotein substrate and an inducer of this protein[104]. This relatively non specific transmembrane protein transports many drugs and other substances out of the enterocyte and back into the intestinal lumen, and it is also expressed on the canalicular membrane of the hepatocyte[104,105]. P glycoprotein expression may therefore be an important determinant of the extent of rifampicin's absorption from the intestine, biliary elimination, and first-pass metabolism and the extent of autoinduction (as it is a

determinant of rifampicin-inducible expression of drug metabolizing enzymes[106]). P-glycoprotein expression in sites such as the blood brain barrier, the blood testes barrier, the placenta, and lymphocyte cell membranes[105,107,108] may also affect the distribution of rifampicin between tissue and cellular compartments of the body.

Less than 50% of the rifampicin dose was recovered in the bile and urine as rifampicin and desacetyl-rifampicin in studies by Acocella et al. More recent evidence suggests that it is likely that the low recovery reflects poor intestinal absorption[28] and/or prehepatic first-pass metabolism[9]. Although an alternative metabolic pathway whereby rifampicin is metabolised to a highly polar glucuronide conjugate has been suggested, the importance of this route has not been confirmed[28].

After a single, 600 mg dose of rifampicin, the 24 hour urinary excretion is about 10% of the dose. The amount excreted in the urine declines with repeated doses to 4 – 6 % of the dose[77]. This is consistent with the finding of increased biliary excretion once autoinduction is established. A derivative of rifampicin, 3-formylrifampicin, is formed spontaneously in the urine especially under acid conditions[59, 28], and confers about 10% of the urinary drug activity.

After a single 600 mg dose the half life of rifampicin is approximately 3.5 ± 0.8 hours. With repeated administration it is reduced to about 1.7 ± 0.5 hours[29,66]. Patients with impaired liver function (due to cirrhosis, chronic hepatitis or acute viral hepatitis) have reduced rifampicin clearance[66,110]. Clearance is also decreased in patients with compromised renal function, however, as the clearance is estimated to be only about 12% of GFR dosage reduction is not recommended[29].

There is a high degree of variability in the initial and induced plasma concentrations of rifampicin. In addition to variations between individuals, significant day-to-day variations are recognized. Variations in rates of drug dissolution, gastric emptying, metabolism or excretion may constitute relevant dynamic effects[28]. Variation in the content and quality of the product ingested may also be contributory. Indeed, as described previously, the requirement for *in vivo* bioavailability testing of rifampicin-containing products is well recognized [30,31,34,111].

Rifampicin is known to induce the activity of several metabolic enzymes including several of the microsomal cytochrome p450 enzymes (CYP3A4, CYP3A5, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and possibly CYP2D6), UDP-glucuronyltransferase, sulphotransferase, the esterase enzyme catalysing rifampicin's

own hydrolysis to 25 desacetylrifampicin and others[90,112,113]. It also induces the expression of the transmembrane efflux pump P-glycoprotein[106]. While rifampicin's interaction with the PXR (pregnane X receptor) orphan nuclear receptor resulting in rapidly increased expression of CYP3A4 is described[12,115], much is still unknown about the metabolic effects of the drug. The clinical relevance of interactions with many drugs via these mechanisms has been established. Systemic concentrations of drug substrates of both P-glycoprotein and CYP3A4 are particularly affected by repeated doses of rifampicin[112]. Especially relevant examples include the antiretroviral protease inhibitors; rifampicin has been shown to dramatically reduce concentrations of lopinavir, saquinavir, amprenavir, indinavir and nelfinavir[116]. As the extent of induction may be dose related, the magnitude of exposure to rifampicin and variability in rifampicin concentrations may be of importance in quantifying the drug drug interactions.

While rifampicin has a dramatic effect on the metabolism of many concomitantly administered drugs, few drug interactions resulting in altered rifampicin levels have been described. The protease inhibitor, indinavir and the commonly ingested piperine (a constituent of black and long pepper) were found in small studies to increase rifampicin concentrations. The AUC of a single 600 mg dose of rifampicin was increased by 73% in 11 HIV infected patients after they received oral doses of 800 mg indinavir 3 times daily for 14 days[117]. The AUC of rifampicin was higher in tuberculosis patients treated with piperine (81 mg.l^{-1}.hr) compared to patients without concomitant piperine (47 mg.l^{-1}.hr)[118]. The elevated rifampicin concentrations may have been as a result of P-glycoprotein and, or, CYP3A4 inhibition[119]. Decreased bioavailability of rifampicin, observed when rifampicin was given concurrently with para-aminosalisylic acid (PAS) has been ascribed to adsorption of rifampicin by the excipient bentonite[90]. The concomitant administration of ketoconazole has been reported to reduce the absorption of rifampicin[82,120], however this finding should be confirmed by further studies .

In a study of the pharmacokinetic interactions between rifampicin, isoniazid and pyrazinamide in untreated patients with pulmonary tuberculosis[121], the concomitant administration of pyrazinamide and isoniazid was associated with an increased clearance of rifampicin reflected by lower AUC values, compared to the concomitant administration of isoniazid alone. As the Cmax values were not significantly different and the reduction in AUC was small, this interaction is unlikely to be of clinical importance.

Isoniazid

Isoniazid is a fundamental component of any first-line antituberculosis regimen. It has more potent early bactericidal activity against *Mycobacterium tuberculosis* than any other known drug and is relatively well tolerated. Although it has excellent activity against actively dividing organisms, isoniazid is only bacteriostatic in the presence of semidormant organisms; it is not a good sterilizing agent[19,55,84]. The drug is taken into the organism by diffusion and oxygen dependent active transport[82]. Isoniazid relies on the mycobacterial enzyme catalase-perioxidase for activation to a moiety that probably inhibits the final steps in mycolic acid synthesis[20,122]. The MIC against *M. tuberculosis* in broth cultures is 0,025 - 0,05 mg/l[20] and the plasma concentrations achieved by administering the standard daily dose (300 mg daily, or approximately 5 mg/kg) are generally reported to exceed the MIC by a wide margin.

Several mutations conferring resistance have been identified. High levels of resistance occur if mutations of the Kat G gene responsible for catalase-peroxidase expression occur. Mutations in the inhA gene are responsible for about 25% of drug resistant isolates. InhA encodes the drug target and mutations are generally associated with lower levels of resistance. Absence of the ahpC gene, like the mutation of katG, appears to interfere with activation of isoniazid. The mechanisms of resistance are unexplained for about 1/3 of isoniazid resistant strains[83,122].

Absorption is reduced when isoniazid is administered with food[73,80]. Peak concentrations of approximately 3 7 mg/l can be expected 1-2 hours after ingestion[56,66,67,82]. Isoniazid is widely distributed to all tissues including the cerebrospinal fluid[20,67,82]. Protein binding is negligible (0-10%)[67]. The half-life is approximately 1.1 ± 0.1 hours and 3.1 + 1.1 hours in fast and slow acetylators, respectively[66,80]. Elimination is largely by renal excretion of acetylisoniazid and isonicotinic acid.

The well described genetic polymorphism of the NAT-2 gene conferring hepatic N acetyltransferase activity is a major determinant of the variability in the pharmacokinetics displayed between individuals, and geographic variation in the distribution of the acetylator type is considerable[66,82, 25]. Slow acetylators have decreased expression of the hepatic enzyme responsible for the conversion of isoniazid into the inactive metabolite N-acetylisoniazid, such that the mean area under the curve from 2 to 6 hours after drug ingestion was 6 times higher than that of fast acetylators in a group of 60 tuberculosis patients in the Western Cape[123]. In the same group of patients a trimodal distribution of isoniazid elimination was

demonstrated with 35%, 45% and 20% slow, intermediate and fast acetylators respectively. The respective half-lives following a 5 mg/kg dose in slow, intermediate and fast eliminators were 3.15, 1.48 and 0.95 hours respectively.

Changes in the acetylator phenotype from fast to slow have been associated with disease progression and wasting in HIV infection[61]. Clearance is also reduced in neonates and in the elderly[66,124].

As the plasma concentrations of isoniazid achieved in patients exceed the MIC for *M. tuberculosis* by a wide margin, a widely held view is that the standardized doses of isoniazid are effective in the vast majority of patients without individualised dosing on the basis of weight or acetylator status. However, some evidence points to a degree of dose dependency in isoniazid's activity: clinical studies conducted by the British Medical Research Council in Madras found a 400 mg daily dose during the second year of treatment was more effective than 300 mg daily in preventing the relapse of disease[19]; and lower isoniazid concentrations and rapid acetylator types are more likely to be associated with inferior treatment outcomes compared to slow acetylators when highly intermittent (once weekly) regimens are used[19,51].

The duration of adequate isoniazid exposure may be more predictive of treatment response than the peak level, as it is thought that bactericidal agents acting on the microbial cell wall exhibit saturable killing at concentrations greater than the MIC[19,56]. Indeed, the AUC seems to be more important than Cmax in once weekly dosing regimens[51]. However, peak concentrations are probably still important for optimal distribution of the drug to the site of action. Studies conducted by the British Medical Research Council in Madras found that once daily doses with 400 mg of isoniazid were more effective than 200 mg twice daily and 200 mg once daily[19]; this finding might support the notion that an adequate peak concentration is important, and together with evidence for the efficacy of intermittent dosing regimens (2 to 3 doses each week) suggests that the drug may exert a significant post-antibiotic effect[63].

Isoniazid is an inhibitor of CYP 2C9, CYP2C19, CYP2D6 and CYP2E1[125,126]. Consequently, the levels of drugs like theophylline, phenytoin, carbamazepine and warfarin might increase when they are used concurrently[27,124]. Induction of CYP2E1 after repeated doses is probably of little importance for pharmacokinetic interactions[126]; but may potentiate the hepatoxicity associated with CYP2E1 substrates like paracetamol, and isoniazid itself.

Several drug-drug pharmacokinetic interactions affecting isoniazid concentrations have been studied: The absorption of isoniazid is diminished by 20-30% when aluminium hydroxide gel is given concomitantly[129]; The concomitant administration of ciprofloxacin may delay the absorption of isoniazid[130]; The antiretroviral drug zalcitabine increased isoniazid clearance in 12 HIV-infected patients, with a consequent reduction in the AUC[131].

Pyrazinamide

Pyrazinamide is a nicotinic acid derivative with useful sterilizing activity against *M. tuberculosis*. Its particular ability to kill organisms in the acid milieu of the macrophage phagosomes confers relapse-preventing properties allowing the duration of treatment to be reduced. Maximal benefit is achieved after 2 months of therapy[19,93,132]. It is used during the intensive phase of treatment, and at lower doses than those used when the drug was first introduced, in order to limit toxicity.

Pyrazinamide is a pro-drug requiring conversion to pyrazinoic acid (POA) by bacillary pyrazinamidase (nicotinamidase). POA is excreted from the microbe by a weak efflux pump, and, under acid conditions, protonated POA is reabsorbed and accumulates in the organism. As the efflux pump is inefficient the metabolite accumulates causing cellular damage. Pyrazinamide is most active against bacilli that are less metabolically active, possibly because the energy dependent efflux pump is less efficient under more stringent growth conditions thereby allowing the metabolite to accumulate more readily[132]. Drug resistance is conferred by mutations in the pyrazinamidase gene, of which several have been described[132].

Pyrazinamide has less variable absorption in patients than rifampicin or isoniazid[142,154]. Peak levels of 30-50 mg/l are attained after a 20-25 mg/kg dose[57]. The drug is displays rapid and almost complete absorption with peak concentrations reached in 1-2 hours. It is widely distributed, has excellent penetration into CSF, and high concentrations have been detected in pulmonary epithelial lining fluid[135]. Protein binding is low (10-20%); the elimination half life is approximately 9.5 hours. The drug is extensively metabolised in the liver, and metabolites are eliminated largely in the urine (about 3% is excreted unchanged)[67]. The pharmacokinetic parameters in adults are reported to be independent of age, sex, height and serum creatinine level, but children appear to have slower absorption and more rapid elimination than adults[133].

Ethambutol

Ethambutol is bacteriostatic at the currently recommended doses. It is added to regimens to reduce the emergence of drug resistance. Optic neuritis is a serious but infrequent side effect of the drug, which is otherwise well tolerated.

There are few published reports of ethambutol concentrations in patients. However, a 2-hour target range of 2-6 mg/l has been proposed[56]. About 75-80% of an ingested dose is absorbed with peak concentrations in the order of 2-6 mg/l within 2-4 hours following a 25 mg/kg dose[57,46]. Absorption is delayed, and peak concentrations and AUC are reduced when ethambutol is taken with food[156]. Minimal (<5%) protein binding occurs. The half-life is approximately 3.1 (±0.4) hours[55]. The drug is eliminated by the kidneys: 75-80% unchanged[44]. Clearance of the drug is therefore reduced in renal impairment.

Streptomycin

Streptomycin may add to the bactericidal activity of regimens during the intensive phase and provide additional benefit in the prevention of the emergence of drug resistance. It is widely used during the intensive phase of treatment for patients who have previously received antituberculosis therapy. Vestibulo-cochlear toxicity and the requirement for parenteral administration limit prolonged use. It is not known to interfere with the pharmacokinetics of the other antituberculosis drugs. Streptomycin concentrations were not measured as part of this project.

II

METHODS

A pharmacokinetic study in a cohort of tuberculosis patients

A prospective pharmacokinetic and pharmacodynamic study was designed to measure the concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol in tuberculosis patients receiving treatment with first line agents; patient and treatment factors that may effect the drug concentrations and the response to treatment. The study commenced in August 1999 after approval of the study protocol (appendix 1.1) by the Research Ethics Committee of the University of Cape Town and an ethics committee convened by the superintendent of Brewelskloof Hospital. The pharmacokinetic observation period ended in February 2002.

The study site

This study was conducted at Brewelskloof Hospital because clinicians based there were interested in the research agenda. The hospital lies on the outskirts of Worcester in the Western Cape and is a referral centre for tuberculosis patients. Patients are referred there primarily from tuberculosis clinics in the Boland/Overberg Region, although patients are also admitted from other health care facilities and more distant locations. Reasons for referral include a poor response to treatment, suspected non-compliance, severe illness or debilitation, complicated disease, poor socioeconomic circumstances and drug resistance. It is a 270-bed hospital with 4 medical doctors and competent nursing personnel. X ray and bronchoscopy facilities are available.

The study population

Eligible patients with pulmonary tuberculosis of 18 years and older, who gave their written informed consent to participate were recruited. Relatively broad study entry criteria were applied such that the study population was representative of the hospital population.

Eligibility criteria

Patients with pulmonary tuberculosis diagnosed by positive sputum smear microscopy for acid and alcohol fast bacilli (AFB), or positive sputum culture for *Mycobacterium tuberculosis*, were included. Eligible patients had received uninterrupted daily antituberculosis therapy, in hospital, with first-line agents (according to regimens for new or retreatment patients: figure 4 Chapter I), for approximately 8 weeks. Critically ill patients, those with contraindications to multiple blood sampling and those

infected with organisms known to be resistant to isoniazid or rifampicin, were excluded.

Sample size

The target sample size was estimated from previous pharmacokinetic studies in patients at DP Marais TB Hospital in Westlake, Cape Town (appendix 2). To detect a mean peak concentration of the rifampicin (the drug with the most variable peak concentrations) in patients responding poorly to treatment that was 25% lower than that of the population mean, 18 poor responders would be required if they comprised 10% of the study population (power 0.8 at the 0.05 level of significance). For the purposes of this calculation a positive sputum culture at 2 months was regarded as a marker of a poor response, and it was estimated by the hospital clinicians and from the literature that approximately 10% of patients would have a sputum culture positive for *Mycobacterium tuberculosis* at 2 months[9]. It was aimed, therefore, to recruit approximately 200 patients.

Clinical study procedure and data collection

Eligible patients who had resided in the hospital for the requisite time were approached to participate in the study. Written informed consent was obtained in the patient's language (Afrikaans or Xhosa) by the attending doctor, with the help of a translator when necessary.

Data pertaining to the patients' history and medical records prior to admission, data from the time of admission, data at 8 weeks after admission, and data up until 2 years after admission were collected. Data was collated in individual patient folders, which were stored in the Division of Pharmacology, University of Cape Town. The data was transferred to electronic files (Microsoft Excel 2002) before analysis.

Data collection forms (appendix 1.3) were used to compile data from each patient by interview and examination. Demographic details recorded included sex and age. Prior histories of tuberculosis and tuberculosis treatment were documented. Past medical history as well as any allergies, chronic or intercurrent illness, and concomitant medication were noted. Regular alcohol use (as reported by the patient and interpreted by the attending clinician), was noted for the year prior to admission to the hospital and during the two months following admission. Similarly, smoking habits and the use of cannabis or other recreational drugs was recorded.

Details of the current episode of tuberculosis were recorded. These included the date of onset of symptoms, the date of diagnosis and the date of hospital admission, the date and results of sputum investigations at diagnosis, and details of any treatment taken before admission to the hospital. Symptoms at the time of admission were sought, and pertinent examination findings on admission (including the weight in kilograms) were documented. A chest x-ray was routinely performed on all patients on admission to the hospital and a single early morning sputum sample was collected for examination (microscopy for AFB and culture of tuberculosis bacilli) except when recent results of sputum investigations sent from the referral center were available. Positive cultures were retained and those of study patients were used to determine the mini-MICs for rifampicin and isoniazid. Limited study resources prohibited validated grading of chest x-ray changes for extent of consolidation and cavitation. Likewise genetic fingerprinting of tuberculosis strains in the sputum to differentiate disease relapse from reinfection was beyond the scope of the study.

At approximately eight weeks following admission serial blood samples were collected to determine the pharmacokinetic profiles of rifampicin, isoniazid, pyrazinamide and ethambutol in plasma; blood samples were collected for haematology, chemical pathology, HIV-serology and immunology evaluation; patients were weighed again; and a single early morning sputum specimen was sent for microscopy and culture to detect *M. tuberculosis*.

After a variable length of time the patients were discharged from Brewelskloof Hospital and referred to clinics near their homes for ambulatory care until treatment completion. Most patients attended the clinics weekly to monthly for collection of their antituberculosis drugs, which were taken with or without the supervision of a designated observer. In accordance with national guidelines, direct microscopy was routinely performed after 5 months of treatment: those patients with two consecutive positive sputum smears were regarded as treatment failures and restarted on intensive phase treatment according to the retreatment regimen.

At the outset of this study it was hoped that the early response to treatment measured by sputum cultures at two months after admission to the hospital would provide an adequate marker for detection of a pharmacodynamic impact of the variability in antituberculosis drug concentrations. Once it became clear that the concentrations of a single drug, rifampicin, were of concern, it was realized that it might be important to measure sterilizing activity more directly. The protocol was

therefore amended to detect treatment failures and relapses in patients admitted to the study after May 2000. Patients were asked to return to the clinic where they received their ambulatory treatment, at 6, 9, 12, 18 and 24 months after their admission to Brewelskloof Hospital. At these visits a sputum specimen was collected and dispatched to the SAIMR laboratory at Green Point for direct smear microscopy to detect AFB and mycobacterial culture. In addition, the clinics were visited by the investigator or designated assistant (when feasible), or contacted telephonically to ascertain the fate of study patients and the results of sputum examinations performed in the routine care of the patient (e.g. Two direct sputum smears were routinely performed at 5 months after treatment initiation and if the clinic staff suspected a worsening or relapse of disease). On study completion, the treatment outcome of each patient, as recorded in the tuberculosis (TB) registers, was documented; and the registers at clinics to which study patients were referred were inspected in order to detect disease relapses (during the period up until 24 months after the last patient was admitted to the hospital). The relevant ethics committees and health authorities approved the amendments.

The protocol, subject consent form, case record form and relevant letters of approval are appended (appendix 1).

Pharmacokinetic assessment

Pharmacokinetic assessment was carried out on each patient after approximately 8 weeks of daily administration of antituberculosis therapy under observation in the hospital. The drug doses were prescribed by the attending physician who used the dosing guidelines in table 1. These guidelines differ slightly from those of the National Tuberculosis Control Programme: they advocate the use of lower rifampicin and isoniazid doses in patients weighing less than 33 kg, and higher doses of pyrazinamide and ethambutol in patients ≥ 33 kg. The drug doses were not altered on the day of pharmacokinetic assessment.

Table 1: Daily doses of rifampicin, isoniazid, pyrazinamide and ethambutol used as a guideline by the prescribing physicians at Brewelskloof Hospital.

Drug	Weight < 33 kg	33 kg ≥ weight < 50 kg	Weight ≥ 50 kg
Rifampicin	300 mg	450 mg	600 mg
Isoniazid	200 mg	300 mg	300 mg
Pyrazinamide	1000 mg	1500 mg	2000 mg
Ethambutol	800 mg	1200 mg	1200 mg

The drug formulations used on the day of pharmacokinetic assessment were those routinely available on the ward medicine trolleys. The product names, batch numbers and expiry dates of each of the formulations used on the day of assessment were recorded.

The concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol were measured in plasma samples from each patient over eight hours following drug administration.

Patients fasted overnight prior to pharmacokinetic sampling. The drugs were administered with approximately 200 ml of water and patients were asked not to eat or drink until a further 2 hours after had elapsed. The drugs were taken under strict observation. The exact times of drug administration were recorded.

Serial venous blood samples were drawn from an arm vein through an indwelling cannula within 1 hour before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 hours after drug administration. The exact times of blood sampling were recorded. Lithium-heparin tubes with a gel separator were used for blood collection. After collection, the samples were immediately placed on ice before centrifugation (3000 rpm for 10 minutes) at room temperature within 30 minutes of collection. From each blood collection tube, 3 plasma samples of at least 1.2 ml were stored in polypropylene tubes on dry ice until transfer to the -80°C freezer in the Division of Pharmacology, UCT, during the evening following sampling. The samples were stored at -80°C until analysis.

Validated HPLC (high performance liquid chromatography) methods developed within the Pharmacology Division were used to determine rifampicin, isoniazid and pyrazinamide concentrations[138] (appendix 3.1). A validated method using mass spectroscopy (LC-MS) was used to determine ethambutol concentrations (appendix 3.2). The fully validated ranges for determination of rifampicin, isoniazid, pyrazinamide and ethambutol concentrations were, 0.3 to 25 mg/l, 0.2 to 15 mg/l, 0.2 to 70 mg/l and 0.1 – 10 mg/l, respectively.

Also on the day of pharmacokinetic assessment, complete urine collections were made from 2 to 4, 4 to 6, and 6 to 8 hours following drug administration. The volume of each urine collection was recorded and two 1 ml aliquots were stored in polypropylene tubes on dry ice until transfer to the -80°C freezer that evening.

The urinary concentrations of the rifampicin were determined by a validated method, which was a modification of the plasma assay method.

Noncompartmental analysis

The raw data comprising plasma concentrations and the corresponding times were stored in Microsoft Excel 2002 before noncompartmental analysis using WinNonlin (version 3.3) to compute the pharmacokinetic parameters and measures.

For the purposes of the determination of the pharmacokinetic measures and parameters, blank values were entered for plasma drug concentrations between 0 and the lower limit of the validated range of determination. Where no deviation from baseline was observed on the HPLC tracing a value of 0 mg/l was assumed. The exact times of blood sampling were used for the determination of the pharmacokinetic measures.

The 5th to 95th centile range of each pharmacokinetic measure was reported in order to reflect the normal range i.e. that range in which the values for 90% of patients lie.

The computational methods used are described below:

Elimination rate constant (K_e or λ_z units; hr^{-1}): First order constant associated with the terminal (log linear) portion of the curve. It is estimated by linear regression of time vs. log concentration.

Half-life ($t_{1/2}$ units: hr): Terminal half-life = $\ln(2) / \lambda_z$.

Lag-time (Tlag; units: hr): the time elapsed from the time of drug administration until the first measurable concentration.

Time of maximum observed concentration (Tmax; units: hr): time elapsed from the time of drug administration until Cmax.

Peak plasma concentration (Cmax; units: mg/l): the drug concentration corresponding to Tmax is the maximum measured drug concentration during a dosing interval.

Area under the curve to the last measurable concentration (AUCt; units: mg.l^{-1}.hr): the area under the concentration-time curve from the time of drug administration until the last measurable drug concentration.

Area under the curve to infinity (AUCi; units: mg.l^{-1}.hr): the area under the concentration-time curve from the time of drug administration, extrapolated to infinity.

$AUCi = AUCt + Clast / \lambda_z$ (Clast = last measurable concentration)

Volume of distribution based on the terminal phase (Vz F obs; units: l):

$Vz\ F\ obs = dose / \lambda_z \times AUCi$

Total body clearance (Cl F obs; units: l.hr^{-1}):

Cl F obs = dose / AUCi

Covariate factors

Patient factors collected by the attending physician and documented in the patient data collection forms after 8 weeks of treatment in the hospital included age, sex, the history of smoking and alcohol consumption in the year before admission (whether, or not, the subject had regularly consumed alcohol - by judgment of the attending physician; and whether, or not, the subject had smoked cigarettes), previous tuberculosis treatment, concomitant illness, concomitant medication, height and weight.

Also at approximately 8 weeks after admission, blood samples were drawn from each patient for chemical pathology (1 x 7 ml clotted tube), serology (1 x 7 ml clotted tube) and haematology (1 x 4 ml EDTA tube and 1 x citrate containing ESR tube) evaluation. The samples were refrigerated at 4°C before transfer within 6 hours to the chemical pathology and haematology laboratories of Groote Schuur Hospital (GSH) and the University of Cape Town (UCT) for analysis. Chemistry included quantification of serum urea, creatinine, total protein, albumin, serum alanine transaminase (ALT), serum aspartate transaminase (AST), alkaline phosphatase (AP), gamma-glutamyl transferase (γ-GT) and total bilirubin. Haematology evaluation included a full blood count (including haemaglobin, mean cell volume (MCV), white cell count (WCC) and platelets), a differential and the erythrocyte sedimentation rate (ESR). The Clinical Virology Laboratory of UCT/GSH/SAIMR (South African Institute of Medical Research) used ELISA to detect HIV 1 and 2 antibodies.

Acetylator genotype and phenotype were determined in a subgroup of 93 patients by a student in the Division of Pharmacology as part of her thesis for her MSc Med[139]. The ratio of N-acetyl isoniazid concentration at 3 hours after drug administration, to isoniazid concentration in the same plasma sample, was used to determine the phenotype. For genotype determination, a sample of blood was collected in EDTA tubes. DNA was extracted and purified from these samples before specific amplification of the NAT2 gene sequences. The amplified DNA was used to investigate the type of polymorphic N-acetyl transferase encoded for each patient. Genotypes were classified as homozygous slow, heterozygous (with slow and fast allele types) or homozygous fast. Although a trimodal distribution the phenotype was identified, for the purposes of this study acetylator status was dichotomized into slow or rapid (which included the intermediate group). There was then excellent agreement between the phenotype and the genotype (heterozygous genotype was included in the fast group). The genotype was used in 4 subjects with discordance between the genotype and phenotype. The phenotype was used in 6 subjects in whom acetylator genotype determination was not successful.

Measurement of treatment response

- *Early response (evaluated 2 months after admission to hospital)*

Weight gain and clearance of *M. tuberculosis* from the sputum are putative markers of the response to treatment[1]. As the result of sputum culture at 2 months is widely regarded as an early measure of the sterilizing activity of a treatment regimen[21], this measure was used for the sample size calculations.

Although the association between a positive sputum culture at 2 months and disease relapse has been demonstrated in several studies[45,47], the determinants of culture conversion at 2 months and the determinants weight gain, sputum smear conversion at 2 months and disease relapse may be different. Furthermore, the relationship of the sputum culture at 2 months to other early response markers and the long term result of treatment may vary between different patient populations. For these reasons additional markers of treatment response were sought.

Each patient was weighed at admission and again after 8 weeks of inpatient treatment. The change in weight as a proportion of the admission weight was documented. Eight weeks after admission, a single early morning sputum specimen

was collected and sent to the SAIMR laboratory in Green Point for direct smear microscopy to detect AFB, and for mycobacterial culture.

• Late treatment response (from 4 until 24 months after hospital admission)

On completion of treatment at the clinics the outcome category was recorded in the tuberculosis registers. The clinic-recorded outcome was categorized according to WHO definitions shown below[7]:

CURED • Initially smear-positive patient who has a negative sputum smear in the last month of treatment, and on at least one previous occasion.

COMPLETED TREATMENT • Patient who has completed treatment but does not meet the criteria for cure or failure.

DIED • Patient who died during treatment, irrespective of cause.

FAILED • Smear-positive patient who remained smear-positive, or became smear-positive again, at least 5 months after the start of treatment.

INTERRUPTED TREATMENT (DEFAULTED) • Patient who did not collect drugs for 2 months or more at any time after registration.

TRANSFERRED OUT • Patient who was transferred to another reporting unit and for whom treatment results are not known.

For the analysis of factors with a bearing on the register recorded outcome, the outcome was dichotomized into successful treatment ('cured' or 'treatment completed') or unsuccessful treatment ('died', 'failed', or 'interrupted treatment'). One observation designated 'transferred out' was omitted from this evaluation.

Furthermore, the outcome of treatment was evaluated, when possible, by regular assessment of sputum samples for the presence of *M. tuberculosis*. Patients were asked to return to the clinic where they received their ambulatory treatment at 6, 9, 12, 18 and 24 months after their admission to Brewelskloot Hospital, so a single sputum sample could be sent to the laboratories of the SAIMR for microscopy and culture. In addition, the clinics were visited by the investigator or a designated assistant, or contacted telephonically to ascertain the fate of study patients.

immunological evaluation

While it is recognized that host immunity is an important determinant of the course of infection and disease caused by *M. tuberculosis*, the correlates of a protective immune response are complex and incompletely characterized. A predominantly cellular response and the type of T-lymphocyte response are important.

In this study, CD4+ CD8+ and CD3 (CD4, CD8) T cell subsets were measured as indications of the cellular proliferative responses. CD4+ suppression is also a marker of the progression of HIV-infection. The cytokine markers were selected to represent expression of type-1 (IFN-γ) and type-2 (IL-5) responses, as well as suppression of type-1 response (IL-10). Plasma levels of each cytokine were measured in addition to the whole blood proliferative responses to stimulation with PPD (purified protein derivative; *Mycobacterium tuberculosis* antigens) and PHA (a mitogen and positive control). The whole blood IFN-γ production after stimulation with *M. tuberculosis* antigens is regarded as one of the most useful and practical methods available to measure a protective immune response to *M. tuberculosis*[142].

At eight weeks after admission a 10 ml venous blood sample was drawn from each patient into a heparinized tube. The sample was kept at room temperature prior to transfer within 24 hours to the immunology laboratory of the Department of Immunology, UCT at Groote Schuur Hospital for analysis of immune markers (appendix 4.1).

The presence of antibodies to Human Immunodeficiency Virus 1 or 2 was determined by ELISA at 8 weeks after admission to the hospital. A tube of clotted blood was kept at approximately 4 °C prior to delivery to the Clinical Virology Laboratory of UCT/GSH/SAIMR (now NHLS) within 24 hours.

Sputum microscopy and culture

Early morning sputum samples were collected and dispatched to the SAIMR laboratories for microscopy and culture.

Direct smear: Prior to staining, the sputum sample was mixed with and equal amount of sodium hypochlorite. The sample was then allowed to stand for 20 minutes to allow killing of viable organisms. After spinning, the supernatant was discarded. A

drop of fixative was placed on a glass slide before adding the precipitate from the spun sputum sample to make the smear. The slide was allowed to dry on a 60 degree hot plate for 20 minutes before staining. Smears of sputum samples were stained for acid fast bacilli prior to microscopic examination (appendix 4.2.1).

Bactec 12B medium was used to culture mycobacteria by the radiometric Bactec method at the SAIMR tuberculosis laboratory in Green Point (appendix 4.2.2).

Drug susceptibility of cultured *M. tuberculosis*

The sputum samples of Browelskloof Hospital patients were routinely sent to the laboratories of the SAIMR in Green Point for culture of *M. tuberculosis*. Those of patients potentially eligible for this study were identified with coloured stickers. Positive culture specimens were retained for determination of rifampicin and isoniazid mini-MICs at the microbiology laboratory of GSH once the patient had been enrolled. Unfortunately, due to logistical problems and administrative errors, most of the sputum culture bottles that should have been saved for mini-MIC determination were lost.

The method for mini-MIC determination (appendix 4.3) allowed determination of the susceptibility of the cultured organisms to 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/l concentrations of isoniazid and 0.06, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/l concentrations of rifampicin using the Bactec system.

Statistical methods

Summary statistics for skewed variables were expressed as medians with centile ranges (a binomial method for obtaining confidence intervals that makes no assumptions as to the underlying distribution of the variable, was used). Nonparametric tests were used for univariate analyses of skewed data, which included most of the pharmacokinetic data. For correlations, Spearman's rank test was generally used. For comparison of a continuous variable between 2 groups within the population, the Kruskal-Wallis equality of populations rank test was used.

Multiple linear regression analysis was used to determine the influence of various patient and drug factors (independent variables, or covariates) on the PK-measures (dependent variables). The following standard procedure was followed:

1. Stepwise backward regression using the full data set with terms removed from the model at a significance level of 0.075, and reentered into the model at significance levels of 0.05.
2. The independent variables selected in step 1 were used in a second regression, to maximize the sample size (only observations for which relevant covariate factor data was missing were not included in the model) the same removal and re-entry levels as in 1 were used.
3. Excluded variables were then reentered one-by-one to see if they were contributory once the number of observations had been maximized.
4. The distribution of the residuals was studied and the presence of multicollinearity was checked; the model was not accepted if a high degree of colinearity existed among the covariates or if the Shapiro-Wilks test showed a skewed distribution. If the degree of colinearity was large the list of independent variables would be studied and rationalized with explanations given and the model building process begun again. If the residuals were skewed, the dependent variable was transformed (by using the natural logarithm, in the first instance; or the square root) and steps 1 and 2 were repeated. If the residuals of the model were still skewed the dependent variable was dichotomized (if this was appropriate) and logistic regression was used.
5. For models with normally distributed residuals, potentially influential observations were identified (using Cook's distance and DFITS) and dropped from the model. If new covariate factors were included in the model without influential observations, these were included in the model applied to the whole data set (including the potentially influential observations unless there was good reason to exclude them). The β-coefficients of the inclusive model were checked to ensure that they did not differ substantially from the original model.
6. Finally, the model assumptions of constant variance, linearity and the appropriate form of the covariates in the model were checked further, using graphical plots of residuals versus fitted values or versus covariate values.
7. Any excluded observations, and the reasons for their exclusion, are reported.
8. In some analyses, certain independent continuous variables were dichotomized (e.g. values of liver function tests were dichotomized in some evaluations into normal and raised).

Certain dependent variables were dichotomized and logistic regression was used to model the covariate effects, either because the continuous dependent variable

could not be described well by the linear regression models, or because there was good reason to categorize the outcome variable (e.g. For Cmax certain covariate factors may be important determinants of drug concentrations below a critical level). The method used to select the independent variables of the final model was similar to that applied for the linear regression analyses. Diagnostic statistics were calculated to identify outliers and influential observations and to assess the validity of the model.

A survival analysis was used to assess the late (2 to 24 month) response. The time to a positive sputum smear or culture, or death (between 2 and 24 months after admission to Brewelskloof Hospital) was used in the analysis, which was censored if the patient was alive and did not have a positive sputum at the time they were lost to follow-up. Cox proportional hazards modeling was used to determine the effect of selected covariates on the relative hazard of an unfavourable outcome with risk variable selection as described below.

Regression approaches were also used to identify factors influencing the measures of response to treatment. Multiple linear regression was used to identify factors determining the percentage weight gain. To identify risk factors for positive sputum microscopy at 2 months, positive early sputum culture, and treatment interruption, treatment failure or death according to the register recorded outcome, multiple logistic regressions were used. To identify covariates influencing the risk of disease recurrence or death, Cox proportional hazards modeling was used. A rationalized approach was necessary for selection of the relevant variables from the vast number covariates.

1. Sub-models were built within each of 3 sub-groups of covariates (those describing patient factors, those describing immune markers; and those describing drug factors including drug levels, dose per kilogram and formulation characteristics); a backward step-wise approach was used whereby variables were removed from the models at a significance level of 0.12 and reentered into the models if $p < 0.10$ to identify potentially important covariates.
2. The covariates from the 3 sub-models describing each response measure were then assessed together in regression analyses using the backwards stepwise approach with limits for inclusion as for the sub-models.
3. The importance of those variables not included in the sub-models by the initial stepwise procedure, but related to the response variable with $p < 0.10$ in univariate analyses, were assessed again (by addition of each variable, one

at a time, to the model), and variables contributing significantly to the model were included.

4. The model assumptions were checked by inspecting the distribution of residuals, and the stability of covariates in the model was checked by removal of potentially influential observations; covariates that did not contribute significantly to the model without potentially influential observations, were removed from the model.
5. The final model included all observations without missing data for the relevant covariates.

Stata Statistical Software (Stata Corporation, USA) releases 7.0 (2001) and 8.2 (2004) were used for most statistical analyses. Statistica (StatSoft, Inc., USA. 1984-2003) was used for most of the graphs generated.

Ethical considerations

Patients were fully informed about the study, including the fact that is would be of no medical benefit to them, and written consent was obtained before enrolment. Voluntary counseling and HIV testing was offered, but not mandatory. Separate written consent was obtained for HIV testing.

As the patients enrolled were studied during their inpatient stay, they were not unduly inconvenienced. Those patients, who returned to the clinics for sputum sampling after completion of their treatment, were remunerated with a food parcel on each occasion.

The Research Ethics Committee of the Health Sciences Faculty, UCT and the local and regional health authorities approved the protocol and protocol amendments.

Insurance and liability

The University of Cape Town subscribes to an insurance policy including liability cover for staff and students engaged in research activities related to their employment or study under the auspices of the university.

Clinical study limitations

This study arose as a result of concerns expressed by the clinicians at Brewelskloof Hospital. For this reason the study was conducted at an outlying site and with a relatively small budget. It was in the nature of the study to disrupt as little as possible the normal processes of admission, investigations, treatment, discharge, subsequent clinic-based care and the work load of the health-care staff. While the observations were, therefore, more likely to reflect the real situation, scientific rigor was compromised in some instances (related in the sections below) by a lack of standardization and disciplined implementation.

Due to major staff changes at Brewelskloof Hospital, the study was terminated before the target number of patients had been recruited. Re-calculation of the required sample size (using the number of culture positive patients at 8 weeks after hospital admission, and the variance of the Cmax of rifampicin form data previously collected in patients at DP Marais Hospital) indicated that the study was adequately powered to detect a 30% difference between the groups. It was therefore decided not to restart the study or set up another site. In retrospect, the greater variability in the rifampicin peak levels in this study decreased the power of the study, such that reduction of 1/3 in the peak concentration of rifampicin could be detected in patients with a poor response to treatment compared to the rest of the population with a power of 0.75, whereas initially we aimed to detect a 25% difference with a power of 0.8. Reductions of 30%, 25% and 18%, for isoniazid, ethambutol and pyrazinamide, respectively, could be detected in those with a positive sputum culture at 2 months with a power of 0.8.

Furthermore, quite by chance, a substantial number of the patients in this study received batches of a substandard rifampicin containing product (associated with more variable plasma concentrations of rifampicin) on the day of pharmacokinetic sampling, thus reducing the power to detect the effect of rifampicin concentrations on outcome and adding complexity to the interpretation of some of the findings.

• *Study population*

The majority of tuberculosis patients in the region receive clinic-based care on an ambulatory basis throughout their treatment course. The variety of reasons that led to admission of patients in this study to the hospital may differentiate them from the

average patient. Caution is therefore warranted in drawing conclusions to be applied to the broader tuberculosis community.

Most eligible patients hospitalized during the period of the study were enrolled. Exceptions included those admitted to one female ward from which no patients were recruited due to staff factors, and patients who were admitted during periods when the collaborating hospital physicians were away. No known biases in selection apart from patient sex (exclusion of females in the above-mentioned ward) occurred because of these factors.

• *Pharmacokinetic data:*

The analytical methods used to determine the drug concentrations in plasma were well established, robust and validated. The laboratory is recognized internationally for its antituberculosis drug assays.

The plasma sample preparation was by a standardized procedure, and there was very little delay before the transfer of specimens onto dry ice. The samples were stored at -80°C until they were assayed.

A relatively intensive blood sampling schedule was used to measure the drug concentrations. The maximal concentrations of the 4 drugs could therefore be reflected reasonably accurately using noncompartmental analysis. For pragmatic reasons, the last blood sample was drawn 8 hours after drug administration; while adequate information regarding the terminal elimination curve of the drugs was generally obtained using this schedule, in some cases with delayed absorption the elimination curve could not be accurately predicted (in a few cases the highest drug concentration was recorded at 8 hours!), and for pyrazinamide, which has a longer half-life, the AUCi was extensively extrapolated.

Pharmacokinetic characterization included only one dosing interval for each patient. The residual variation (not attributed to measured covariate factors) in drug concentrations within individuals ['inter-occasional variation'; 'intra-individual variation'; IOV) could therefore not be evaluated. It is important to recognize that if IOV is great, pharmacokinetic measurements from one occasion may not be representative of the drug exposure over the treatment period, and drug concentration monitoring on a single occasion is less likely to predict the response to treatment.

• *Covariate factor data:*

A relatively heterogeneous population of patients was included in the analysis. The covariates collected were by no means comprehensive of all details which may have implications for the pharmacokinetics of the 4 drugs measured. Relatively invasive, expensive and esoteric investigations were avoided: although gastro intestinal pH putatively influences rifampicin absorption[35] it was not measured: although emerging literature points to the potential pharmacokinetic relevance of drug transporting and metabolizing enzymes for many drugs, investigation for the presence of polymorphisms or quantifying the expression of these enzymes was well beyond the scope of this project (besides characterization of acetylator status in a subgroup of the patients).

While for most covariate factors relatively complete data was gathered, incomplete data for some covariates (eg. acetylator status was determined in subset of patients only) decreased the power of some assessments.

Patient history was relied upon for gathering information about previous medical history, concurrent symptoms such as diarrhoea, smoking habits and the consumption of alcohol and other drugs. For pragmatic reasons most of this data was not verified by other sources.

While the drug formulations used and their batch numbers were noted the date of manufacture was not recorded and shelf conditions subsequent to manufacture were not known. Recent literature suggests that the degradation on the shelf of rifampicin (in particular) and isoniazid may be substantial, especially in fixed dose combinations and under conditions of high temperatures and humidity[37].

• *Factors influencing treatment response:*

The response to treatment of tuberculosis patients is likely to be the consequence of multiple interacting and ill-defined factors. This study focused on the importance of drug concentrations; however it is likely that much of the variability in treatment response is determined by other factors, and these were not comprehensively qualified or quantified.

Treatment adherence is arguably the most important determinant of the response to a treatment regimen. Compliance was assumed to be excellent during the period of

hospital admission where treatment was given under direct observation. However, there was very little control over the manner in which treatment was dispensed, taken or recorded once the patients were discharged for ambulatory care. Clinics varied with respect to the nature and reliability of drug dispensing data and most patients did not receive clinic-based DOTS. Although adherence could potentially account for much of the variability in treatment outcome and disease relapse, assessment of treatment adherence was outside the scope of this study.

The study population included a diverse patient group: those on tuberculosis treatment for the first time together with patients with a history of previous disease or multiple relapses; the spectrum of disease severity was wide; some patients had concomitant illness; immunity was variable. While extensive characterization of patient profiles was attempted, accurate quantification of all the factors relevant to treatment outcome was impossible within the confines of the study. Adjustment of treatment outcome for patient factors was therefore crude.

Correlates of a protective immune response are complex and not yet fully understood; assessment of immune status in this study was superficial. Immune measurements focused on the cytokine response to stimulation of whole blood with purified protein derivative of *M. tuberculosis*. While certain cytokine responses are thought to be important for immunity against *M. tuberculosis*, they comprise only part of the protective response. Furthermore, interpretation of the role of the cytokines is complicated: for example, IL-10 (a marker of TH2-type response) may enhance the ability of dendritic cells to inhibit intracellular growth of mycobacteria[41]. Furthermore, financial and practical constraints dictated that only a limited number of cytokine responses could be measured. Cytotoxic T cell subsets (CD4+, CD8+ and CD3+ lymphocytes) were quantified but the cytotoxic capacity of effector cells was not measured. Finally, it should be noted that the levels of markers of immunity change with time during the course of antituberculosis treatment: the responses measured after 8 weeks of treatment in hospital provides a mere glimpse of patient immunity at one point in time.

While presence of HIV-infection was detected, viral load was not quantified due to budgetary constraints. As only approximately 10 % of the study patients were HIV-infected, the power of the study to determine the influence of this factor was limited.

The dietary supplements vitamin A and zinc may improve the response to treatment in depleted patients[8]; nutritional status and diets were not evaluated in this study.

The extent of cavitation on chest x ray is associated with disease relapse. In this study cavities were not routinely assessed as available personal lacked experience for this evaluation.

Variability attributed by the pathogen was also incompletely assessed. While the susceptibility of the *M. tuberculosis* isolates to isoniazid and rifampicin were measured in some patients by determination of the mini-MICs, in most patients no such assessment was made; and no measure of virulence was feasible.

Another important source of variability is introduced by the variable length of treatment received by patients prior to hospital admission. While many patients were admitted within 2 weeks of diagnosis, those admitted for reasons of poor treatment adherence or poor clinical response had generally been receiving clinic-based treatment for longer periods.

• *Measurement of treatment response:*

The sensitivity of the technique for the detection of AFBs by direct microscopy is relatively low: probably around 30 – 40% if a single specimen is examined, increasing to 65 –75 % with multiple specimens[142] and varies according to the organism burden, the method used for specimen preparation, the skill of the observer and the time to review each slide. Furthermore, rapidly growing mycobacteria vary in their ability to retain acid-fast stains. The direct smear is reported to have a specificity of > 99% and a positive predictive value of 91.5 - 98% for the diagnosis of tuberculosis [4]. However, smear positivity may be conferred by artifacts, non–viable *M. tuberculosis* (especially in patients already taking anti-tuberculosis therapy) and mycobacteria other than tuberculosis.

Mycobacterial culture is more sensitive and specific than is the direct smear for AFBs. Sensitivity and specificity rates for pulmonary tuberculosis have been estimated at 81% and 98.5%, respectively[142]. The sensitivity is increased to approximately 96% for cavitary disease[142]. False negatives may occur if cultures loose viability and contamination may result in false positive results.

Due to cost constraints, only single sputum specimens were sent for bacteriological evaluation in this study, thereby decreasing the sensitivity of the investigations.

For reasons cited above the smear and culture findings are not necessarily concordant. In this study the culture result at 2 months was used as the primary marker of early treatment response. While culture positive sputum 2 months after treatment initiation is associated with an increased risk of unfavourable treatment outcomes, a high proportion of those with a positive sputum at this time will be cured by short course chemotherapy[4], such that sputum culture at 2 months has a low specificity to predict the sterilizing activity of treatment in an individual. It is unclear how accurately the markers of early treatment response reflect improvement in the tuberculosis disease.

Sterilizing activity is best reflected by the rate of disease recurrence after an initial response (late treatment failure or relapse of disease after completion of treatment). Detection of disease relapse was not standardized in all cases as many of the scheduled sputum specimens after treatment completion were not available. Firstly, as mentioned previously, the protocol was amended to include the collection of data relating to the patients' progress after discharge from the hospital, only after several patients had already been recruited. Secondly, implementation of clinic-based follow-up was onerous: The clinics where patients received their ambulatory treatment were scattered over a huge area (appendix 7). Individual home visits for collection of sputum samples and ascertaining the well-being of patients after treatment completion were not possible without placing unreasonable demands on clinic staff. Patients were therefore relied on, perhaps unfairly, to return to clinics at the allotted times of their own accord.

Disease relapse could not be differentiated from re-infection in this study as strain comparison (eg. by restriction fragment length polymorphism analysis) was not done.

Early bactericidal activity (EBA, a marker of the activity of drugs against those rapidly dividing organisms in the aerobic environment of pulmonary cavities) was not measured in this study. The importance of drug concentration on EBA was therefore not assessed.

While weight change is used by physicians on a daily basis to assess the response of tuberculosis patients to treatment, it is confounded by many extraneous factors and is not a validated tool.

III

THE PHARMACOKINETICS OF RIFAMPICIN, ISONIAZID, PYRAZINAMIDE AND ETHAMBUTOL

In this chapter the pharmacokinetic characteristics of rifampicin, isoniazid, pyrazinamide and ethambutol in the study cohort are described.

Serial blood samples were drawn on one occasion in 143 patients receiving first line treatment regimens two months after their admission to Brewelskloof Hospital. Drug concentration-time profiles and pharmacokinetic measures were determined by previously described methods. One patient was excluded, as he did not meet the eligibility criteria (the diagnosis of tuberculosis was never verified by microscopy or culture). The results from 142 patients are presented.

The blood samples were drawn at times very close to the planned times. The 4-, 6- and 8-hour plasma samples of one subject were not available for analysis. The actual time of blood sampling was recorded to the nearest minute. Table 1 summarizes the exact time from drug ingestion to sampling for each of the planned sampling times. On the whole, the exact times of sampling deviated very little from the planned time.

Table 1: Exact sampling times: Blood samples were drawn within 1 hour prior to drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 hours following drug administration; the exact times of blood sampling were recorded.

Time (hrs)	N	median exact time[2] (hrs)	Centiles (hrs)		Range of deviation from planned time (hrs)
			5th	95th	
0.5	142	0.50	0.50	0.64	0.16 to 0.26
1.0	142	1.00	1.00	1.08	-0.02 to 0.70
1.5	142	1.50	1.50	1.55	-0.02 to 0.17
2.0	142	2.00	1.98	2.06	-0.02 to 0.16
2.5	142	2.50	2.50	2.55	0.02 to 0.27
3.0	142	3.00	2.98	3.06	-0.03 to 0.60
4.0	141	4.00	4.00	4.05	0.02 to 0.13
6.0	141	6.02	6.00	6.11	-0.02 to 0.27
8.0	141	8.00	7.98	8.13	0.03 to 0.24

[1] planned time (hours) of blood sampling after drug administration
[2] number of patients sampled. 4-, 6- and 8- hour plasma samples for one patient were lost
[3] actual sampling time

The exact sampling times and corresponding plasma drug concentrations for each subject are presented in Appendix 5.1.

RIFAMPICIN

A group of 54 of the patients received (on the day of pharmacokinetic monitoring) batches of single drug rifampicin formulations, which were subsequently withdrawn by the national medicines regulatory authority, as sufficient bioavailability data had not been submitted to the authority after what the manufacturer had considered to be minor formulation changes. Importantly, the results of dissolution testing of the substandard formulations (provided by the manufacturer) that were taken by

patients in this study met the regulatory requirements (appendix 8); comparative results of dissolution testing for the approved products used in this study are not available.

Assuming the release of rifampicin-containing products not conforming to the regulatory requirements is infrequent; the pharmacokinetic properties measured when the approved formulations were used may be more representative than those of the entire cohort. The pharmacokinetic measures for rifampicin derived after ingestion of the fully approved formulations are therefore summarized separately from those of the drug batches not approved by the regulatory authority. Other covariate influences are reported in the next chapter.

The pharmacokinetic measures of rifampicin derived from the individual concentration-time profiles are tabulated below (Table 2).

Table 2: The elimination rate constant (k), half-life, lag time, time to reach the peak concentration (Tmax), the peak concentration (Cmax), the area under the concentration-time curve to the last measurable concentration (AUCt), the area under the concentration-time curve to infinity (AUCi), the volume of distribution (Vz F obs) and the clearance (Cl F obs) of rifampicin, for each subject, 2 months after admission to Brewelskroof Hospital. The values for subjects who received products not approved by the regulatory authority are highlighted in yellow

Subject	K (hr^{-1})	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg.l^{-1})	AUCt (mg.l^{-1}.hr)	AUCi (mg.l^{-1}.hr)	Vz F obs (l)	Cl F obs (l.hr^{-1})
1	0.41	1.71	0.0	2.52	12.49	46.65	49.82	22.22	9.03
2	0.28	2.43	2.0	4.0	5.14	12.15	14.96	68.67	18.08
3	0.28	2.46	0.5	3.0	4.26	21.37	25.96	62.01	17.33
4	0.50	1.37	0.5	1.5	4.63	15.26	16.73	53.33	25.90
5	0.39	1.78	0.0	3.0	5.29	19.11	23.34	65.99	25.71
6	0.39	1.79	0.0	1.5	3.76	12.61	14.89	104.61	40.55
7	0.32	2.16	1.0	2.5	3.65	25.08	30.04	46.77	14.98
9	0.26	2.70	0.0	2.0	14.84	65.80	80.08	29.9	7.49
10	0.39	1.76	0.0	2.5	8.56	28.58	31.86	35.85	14.12
11	0.70	0.99	0.0	2.0	6.74	20.3	21.09	30.48	21.43
13	0.47	1.47	1.5	3.0	8.53	20.85	22.50	42.39	20.00
14	0.23	3.01	0.0	2.5	4.41	21.16	26.63	97.63	22.51
15	0.24	2.94	.0	2.0	8.13	35.91	44.80	42.59	10.05
16	0.71	0.97	0.0	2.5	2.54	5.36	6.62	95.42	67.93
17	0.27	2.52	0.0	3.0	8.41	39.31	47.87	34.27	9.41
18	0.41	1.71	0.0	2.02	8.21	32.02	34.61	32.05	13.00
19	0.57	1.22	0.5	2.67	9.04	27.28	28.00	28.25	16.07
20	0.28	2.51	0.0	1.5	14.91	63.55	71.29	22.84	6.31
22	0.38	1.85	0.0	2.02	14.04	47.21	50.75	30.66	11.82
23	0.15	4.74	0.0	3.0	12.51	48.31	74.88	54.78	8.01
24	0.44	1.57	0.5	2.5	2.51	9.63	10.36	98.68	43.45
25	0.23	2.98	0.0	2.52	12.31	58.70	74.52	25.94	6.01

Table 2 continued

Subject	F (hr⁻¹)	$t_{1/2}$ (hr)	t_{lag} (hr)	t_{max} (hr)	C_{max} (mg/L)	AUC (mg/L·hr)	AUMC (mg/L·hr²)	Vz F obs (L)	CL F obs (L·hr⁻¹)
26	0.30	2.31	0.5	2.0	4.88	22.63	76.13	76.51	22.96
27	0.20	3.50	0.5	2.98	0.91	4.53	6.55	346.94	68.74
29	0.46	1.49	0.0	1.0	1.92	7.24	8.10	119.83	55.57
30	0.30	2.31	0.5	2.98	1.64	6.23	7.36	205.46	61.14
31	0.50	1.38	0.5	2.52	12.11	36.99	38.40	23.25	11.72
34	0.38	1.84	0.0	2.52	3.58	13.65	14.91	90.02	30.17
37	0.45	1.52	1.5	3.0	2.94	11.50	12.42	79.44	36.04
41	0.30	2.30	0.0	1.05	2.09	5.31	6.49	307.12	92.46
42	-	-	1.05	8.0	0.73	3.16	-	-	-
43	-	-	-	-	0.00[c]	0.00[c]	-	-	-
44	0.30	2.33	0.44	2.0	11.60	44.81	52.06	29.10	8.64
45	0.40	1.72	0.0	2.5	1.42	5.20	6.02	185.50	74.69
46	0.34	2.03	0.0	3.0	6.34	20.68	23.66	55.57	19.00
47	0.32	2.16	0.0	2.5	5.84	24.46	27.75	50.60	16.25
48	0.05[a]	13.31[a]	0.0	4.0	3.74	17.24	67.78[a]	169.92[a]	8.85[a]
49	0.53	1.32	0.0	1.58	1.68	4.41	5.57	204.49	107.75
50	0.22	3.19	0.0	2.0	20.41	81.62	102.67	20.16	4.36
51	0.31	2.22	0.0	4.0	4.59	21.47	25.62	75.15	23.47
52	0.23	3.08	0.5	2.52	8.21	36.48	48.33	41.34	9.31
53	0.46	1.46	0.0	3.0	4.62	18.12	18.96	66.47	31.64
54	-	-	-	-	0.00	0.00			
55	0.42	1.64	1.5	2.52	11.63	28.62	31.15	34.20	14.45
56	0.34	2.01	0.0	2.5	7.12	29.43	32.07	40.72	14.03
57	0.40	1.71	1.0	2.5	4.57	15.88	17.39	54.01	25.88
58	0.28	2.51	0.52	2.5	4.72	17.76	20.49	79.46	21.96
59	-	-	1.08	4.0	0.60	1.17			-
60			-	-	0.00[c]	0.00[c]			
61	0.42	1.65	0.0	2.5	2.04	4.59	7.56	147.89	62.01
63	0.28	2.43	0.0	1.98	5.26	24.61	29.67	71.01	20.22
64	0.38	1.81	0.0	1.07	4.56	15.61	16.63	94.49	36.09
65	0.05[a]	15.14[a]	0.0	1.0	0.83	3.77	34.59[a]	674.00[a]	30.85[a]
66	0.19	3.71	0.0	2.02	10.65	48.87	66.59	48.28	9.07
67	0.70	0.99	0.5	3.0	3.24	7.82	8.40	76.45	53.57
68	-		1.5	8.05	0.77	3.22	-	-	
70	-	-			0.00[c]	0.00[c]	-	-	
71	-	-	3.05	8.02	3.33	5.05	-		-
72	0.43	1.60	0.0	1.5	8.43	28.45	30.21	34.48	14.89
73	0.34	2.05	0.5	2.5	1.17	3.06	3.96	336.95	113.69
74	0.46	1.49	1.0	2.52	6.49	14.76	17.33	55.92	25.96
75	0.42	1.67	0.48	1.52	4.26	13.37	14.31	75.63	31.45
77	0.45	1.53	0.0	1.0	7.78	31.69	33.63	39.48	17.84
80	0.31	2.27	1.0	2.5	9.14	29.73	36.02	54.52	16.66
81	0.29	2.39	0.0	1.98	11.58	49.78	57.90	35.68	10.36
83	0.48	1.44	0.0	1.0	2.62	9.80	10.94	114.10	54.85
84	0.32	2.16	0.0	1.08	17.46	51.52	56.84	24.62	7.92

Table 2 continued

Subject	K (h⁻¹)	half life (h)	lag time (h)	Tmax (h)	Cmax (mg/l)	AUCt (mg·h/l)	AUCt (mg·h/l)	Vz F obs (l)	Cl F obs (l/h)
86	0.39	1.77	1.02	4.0	3.91	16.14	18.22	63.15	24.69
87	-	-			0.00	0.00			-
88	0.25	2.79	0.0	1.5	13.97	44.24	51.84	34.92	8.68
89	0.38	1.83	1.5	4.0	6.55	21.73	25.47	46.53	17.67
90	0.47	1.49	0.0	4.0	6.26	22.87	24.92	51.59	24.08
91	-	-	1.5	6.0	1.54	6.37			-
92	0.46	1.49	0.5	2.5	2.40	7.35	8.43	153.23	71.17
93	0.10	6.76	0.0	2.52	7.98	40.98	83.40	52.66	5.40
94	0.29	2.35	1.0	2.52	2.62	9.35	11.16	136.77	40.92
95	-	-	0.0	3.03	0.85	2.17	-	-	
96	0.60	1.15	0.0	2.5	7.57	25.11	25.74	38.64	22.31
97	0.56	1.24	0.0	2.5	13.11	31.73	32.70	24.56	13.76
101	0.63	1.10	0.0	2.0	4.04	12.55	13.23	71.98	45.36
103	0.35	1.98	0.0	2.0	6.74	21.71	24.16	70.92	24.84
104	0.42	1.64	0.58	2.5	6.36	20.32	22.02	64.39	27.25
105	0.62	1.11	0.52	3.0	5.35	17.16	17.67	40.87	25.46
106	0.26	2.67	2.52	4.02	1.04	4.21	5.62	307.62	80.01
107	0.27	2.56	0.0	1.5	5.57	24.07	28.24	58.87	15.93
108	0.50	1.37	0.0	2.5	5.19	18.64	19.55	45.67	23.02
109	0.51	1.35	0.0	1.5	3.65	11.57	12.57	69.98	35.80
110	0.30	2.34	0.0	2.55	2.64	10.61	12.38	122.80	36.34
111	0.52	1.35	0.0	3.0	4.48	14.53	15.25	57.29	29.30
112	0.29	2.42	0.0	2.5	4.82	21.02	24.27	64.79	18.54
113	0.41	1.71	0.0	1.5	5.83	24.11	25.49	58.00	23.53
115	0.38	1.82	0.0	1.52	3.72	13.87	14.91	79.30	30.19
117	0.34	2.04	0.0	2.03	5.22	20.02	22.32	59.27	20.16
118	0.25	2.83	0.0	1.98	5.57	24.61	30.70	79.66	19.54
119	0.64	1.59	0.2	1.02	3.60	11.11	12.51	110.04	47.95
120	-	-	0.0	2.02	6.72	-	-	-	-
121	0.29	2.40	0.0	2.5	6.38	28.15	33.84	45.97	12.30
122	0.39	1.80	0.5	2.5	3.11	10.85	12.21	95.42	36.84
123	0.23	3.00	0.52	3.0	5.75	21.53	27.93	69.74	16.11
124	0.37	1.90	1.58	3.03	6.35	24.00	15.85	77.66	28.36
125	0.38	1.80	3.0	7.55	2.30	8.56	10.16	115.12	44.30
126	0.32	2.15	0.0	1.05	7.70	31.41	34.69	40.27	12.97
127	0.33	2.13	0.53	4.02	4.74	19.40	23.34	59.28	19.28
128	0.44	1.56	0.0	1.53	3.08	12.25	12.94	104.53	46.36
130	0.30	2.32	0.0	2.52	6.50	22.92	26.26	57.45	17.14
131	-	-	0.5	6.0	3.29	17.63	-	-	-
132	0.35	1.96	0.0	2.5	3.31	13.77	15.44	82.43	29.15
133	0.39	1.79	0.0	2.52	5.27	19.04	20.78	74.59	28.88
134	0.43	1.62	0.0	2.0	11.92	55.22	59.10	17.80	7.61
135	0.46	1.43	2.5	4.0	4.09	10.28	11.61	80.21	38.77
136	0.53	1.32	0.0	1.5	5.28	17.76	19.12	59.51	31.36

Table 2 continued

Subject	Ke (hr-1)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/l)	AUCt (mg/l*hr)	AUCinf (mg/l*hr)	Vz F obs (l)	Cl F obs (l/hr)
137	0.54	1.29	0.0	2.52	4.93	15.33	16.00	52.23	28.13
138	0.25	2.82	0.0	2.0	13.52	52.01	65.54	37.31	9.15
139	0.45	1.53	0.5	2.5	10.92	44.97	47.19	21.07	9.54
141	0.34	2.03	0.0	2.0	8.45	36.25	39.58	44.39	15.16
142	0.28	2.50	0.0	1.67	6.26	21.28	24.69	65.77	18.23
143	-	-	0.0	8.0	1.32	2.64	-	-	-
144	0.27	2.54	0.0	1.84	4.01	19.40	21.03	78.44	21.40
145	0.34	2.03	0.5	2.98	4.35	16.97	19.20	68.49	23.44
146	0.25	2.82	0.5	2.53	11.70	38.14	48.54	90.28	12.36
147	0.19	3.65	0.0	1.58	6.65	31.51	41.52	57.11	10.84
148	0.23	3.00	0.0	2.66	5.30	28.49	35.83	54.30	12.56
149	0.28	2.46	0.53	3.03	3.29	12.95	16.20	98.75	27.77
150	0.50	1.38	0.5	1.5	8.41	24.06	24.85	48.05	24.14
15	0.44	1.58	0.0	1.5	7.24	27.31	28.51	35.94	15.78
152	0.38	1.81	0.0	2.0	5.87	25.61	27.20	43.18	16.54
153	0.19	3.65	0.0	4.0	4.80	20.96	31.61	74.91	14.24
154	0.76	0.92	0.0	2.53	1.30	32.05	33.02	18.04	13.63
155	0.31	2.24	0.5	2.0	11.1	41.89	42.84	40.58	12.54
156	0.39	1.81	0.6	3.0	9.35	31.02	34.15	46.83	17.57
158	0.63	1.31	0.0	1.0	5.70	20.40	22.19	51.10	27.04
159	0.27	2.58	0.0	2.97	8.26	36.15	46.50	47.97	12.90
160	0.47	.46	0.0	3.0	3.08	8.07	9.53	99.76	47.20
161	0.56	1.24	0.0	2.5	6.29	23.58	25.41	31.73	17.71
162	0.37	1.86	0.0	2.52	8.3	36.90	38.41	36.07	13.47
163	0.18	3.97	0.0	2.5	6.59	33.03	49.00	61.58	9.15
164	0.30	2.29	0.0	2.53	9.25	44.05	50.63	39.12	11.85
165	0.54	1.28	0.0	2.5	4.17	10.44	11.56	71.89	38.93
166	0.63	1.11	0.0	2.5	3.96	10.51	11.17	64.34	40.28
167	-	-	0.5	6.03	2.77	10.94	-	-	-
168	0.43	1.61	0.0	2.5	3.73	12.16	14.49	75.95	41.41
169	0.21	3.29	0.0	2.5	9.27	39.10	51.16	41.69	8.80

	Ke (hr-1)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/l)	AUCt (mg/l*hr)	AUCinf (mg/l*hr)	Vz F obs (l)	Cl F obs (l/hr)
Data summary									
n	125	125	137	137	142	141	125	125	125
median	0.38	1.83	0.0	2.50	5.28	23.31	24.92	57.45	20.22
5th centile	0.19	1.11	0.0	1.02	0.74	2.26	6.57	27.96	7.70
95th centile	0.63	3.60	1.5	6.00	13.08	51.35	69.88	198.07	70.44
	Ke (hr-1)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/l)	AUCt (mg/l*hr)	AUCinf (mg/l*hr)	Vz F obs (l)	Cl F obs (l/hr)
Data summary for fully approved formulations (54 observations excluded)[1]									
n	59	83	89	89	89	87	83	83	83
median	0.37	1.86	0.0	2.50	5.89	21.47	25.62	57.45	19.54
5th centile	0.19	1.10	0.0	1.02	2.35	6.16	8.65	22.35	8.17
95th centile	0.63	3.65	1.28	4.01	13.24	51.17	66.33	114.91	65.31

Table 2 continued

	K (hr⁻¹)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/L)	AUC1 (mg/L.hr)	AUCI (mg/L.hr)	Vz F obs (L)	CL F obs (L/hr)
Data summary for product batches not approved by the regulatory authority[d]									
n	42	42	49	49	54	54	42	42	42
median	0.38	1.82	0.0	2.52	3.82	13.73	18.89	58.08	23.86
5th centile	0.20	1.13	0.0	.64	0	0	5.68	23.44	5.49
95th centile	0.6	3.82	2.01	8.01	13.32	53.32	82.07	332.55	79.21

Missing pharmacokinetic measures could not be calculated due to insufficient measurable drug concentration points.

[d] Data excluded from further analysis because the goodness of fit of the elimination rate constant to the data is poor (subject number 48 rsq=0.80; 65: rsq=0.11); and the values of K and half-life are, as a result, outliers

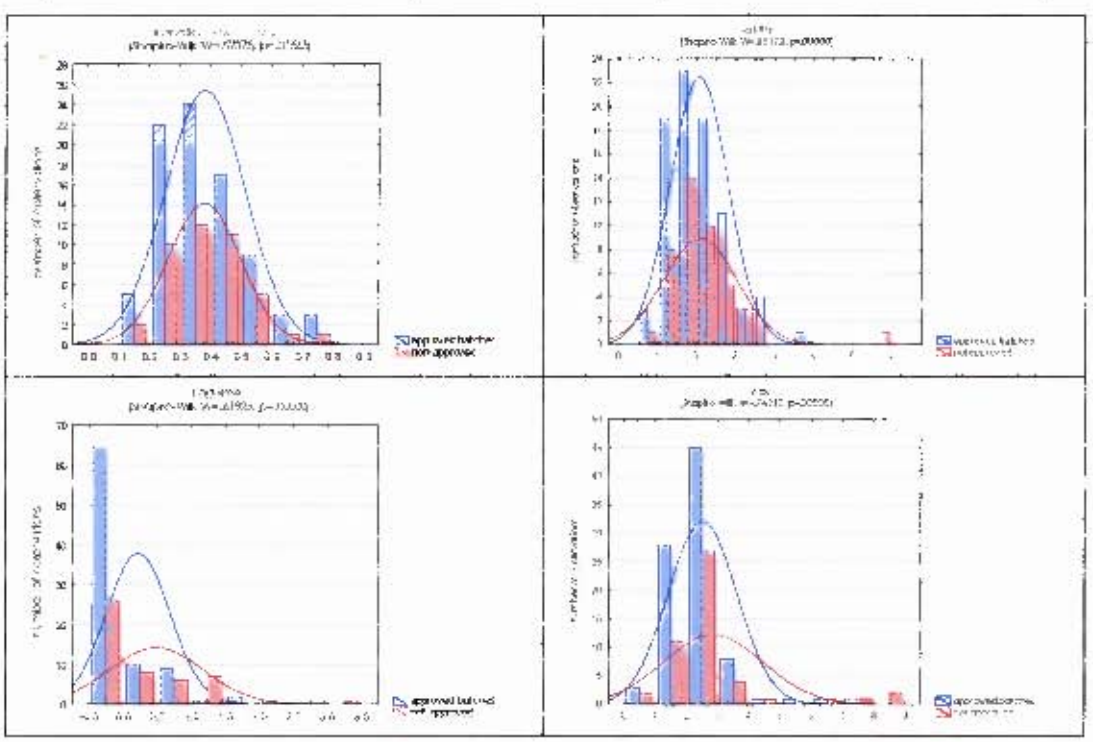
[e] Data excluded from further analysis as sampling was only available until 3 hours after drug administration.

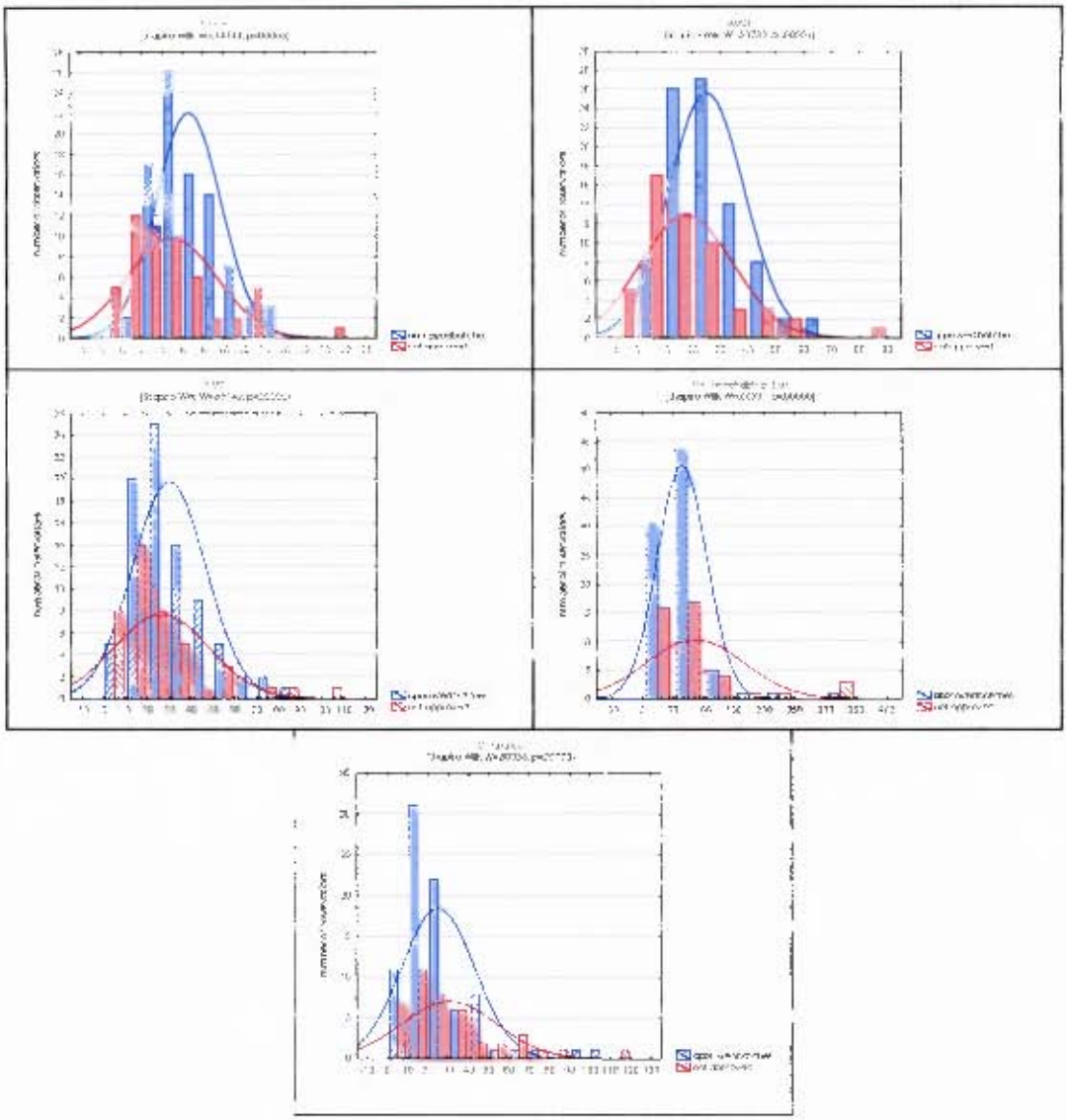
[f] Cmax and AUC1 value of 0 mg/l was assigned to these cases (43, 60 and 70) where the peak concentrations were not measurable as they fell below the validated range of the assay.

[g] Fifty four patients were exposed to specific batches of formulations that were subsequently withdrawn by the national drug regulatory authority, as sufficient bioavailability data had not been submitted after what the manufacturer had considered to be a minor formulation change. (see Appendix 8 for further details)

The distribution of the data for each pharmacokinetic measure is illustrated in figure 1. The Shapiro-Wilk test for normality showed that the data were skewed for all the pharmacokinetic measures, except the elimination rate constant[h]. The elimination rate constant was skewed when only the data from fully approved formulations was used.

Figure 1: Histograms for each pharmacokinetic measure of ciprofloxacin show the distribution of the data in 142 patients (values measured in patients who took drug batches approved by the regulatory authority are represented in blue; those measured in patients who took non-approved batches are represented in red).





Relatively intensive blood sampling (every 0.5 hours for the first 3 hours after drug administration) allowed reasonably accurate determination of the peak rifampicin concentration in most patients. Peak concentrations were attained from 0.5 to 2.5 hours in 98 cases (72% of the 137 cases where Tmax was determined).

The sampling schedule duration was adequate for detecting the overall drug exposure in most patients. In 17 of the 142 patients insufficient measurable drug concentrations were obtained for the determination of the elimination rate constant. Of the remaining 125 profiles, only 23 (18%) had a ratio of AUCt to AUCi of less than 0.80 and only 8 (6%) of these profiles had a ratio of less than 0.75.

The median peak rifampicin concentration was 5.28 mg/l (95% confidence interval (CI) of the median: 4.62 to 6.01), and the median time to reach the peak

concentration was 2.5 hours (95% CI: 2.50 to 2.52). The median peak concentration was significantly lower for the product batches not approved by the regulatory authority (3.82 mg/l: 95% CI: 2.63 to 5.36), compared to that for fully approved products (5.89 mg/l: 95% CI: 5.00 to 6.53); p=0.0011. The peak concentration also appeared to have a bimodal distribution for the non-approved products (Figure 1). The time to reach the peak concentration was not significantly longer for the non-approved batches (p = 0.2632), however, the lag time was greater for these batches (p = 0.0096), with 43% of the non-approved batches having measurable lag times vs. 26% for the approved products.

It has been suggested that, for rifampicin, the most important pharmacokinetic marker of activity is the Cmax : MIC ratio (or perhaps, the proportion of the AUC above the MIC). The proportions of patients with maximal plasma concentrations below 8 mg/l (the lower limit of the published reference range for the peak concentration[5,6,7]); 4 mg/l (the lower limit of the normal range quoted in some references[8]) and 2 mg/l are summarized in table 3.

Table 3: The frequency of Cmax concentrations below 8mg/l, 4mg/l and 2mg/l.

Cmax	<2 mg/l	<4 mg/l	<8 mg/l
All products (n=142)	19 (13.4%)	47 (33.1%)	105 (73.9%)
Approved products (n=88)	2 (2.3%)	19 (21.6%)	61 (69.3%)
Non approved batches (n=54)	17 (31.5%)	28 (51.85%)	44 (81.5%)

The proportion of cases in which the peak concentration fails to reach the lower limits of the recommended ranges is cause for concern. While the non-approved batches performed particularly poorly with over 50% of the Cmax values lying below 4 mg/l, for the approved products over 20% of cases had peak concentrations below this level.

The median AUC1 and AUC4 values were 20.31 mg.hr.l^{-1} (95% CI: 17.39 to 21.65) and 24.92 mg.hr.l^{-1} (95% CI: 22.07 to 27.17), respectively. As for the Cmax, the AUC values were lower for those product batches not approved by the regulatory authority 13.73 mg.hr.l^{-1} (95% CI: 8.24 to 18.39) vs. 23.47 mg.hr.l^{-1} (95% CI: 20.18 to 24.61), p =0.0004, for AUC1; and 18. 89 mg.hr.l^{-1} (95% CI: 14.91 to 27.66) vs. 25.62 mg.hr.l^{-1} (95% CI: 23.41 to 30.01), p=0.0557, for AUC4.

The median concentration-time curve for rifampicin (figure 2.1), with error bars depicting the range of concentrations at each sampling time and the boxes representing the 25th to 75th percentiles, illustrates the extensive variability of the concentrations of this drug in the study patients; with the highest variability in

rifampicin concentrations displayed in those patients who received non-approved products (Figure 2.2).

Figure 2.1: The median concentration vs. time curve for rifampicin in all patients (n=142).

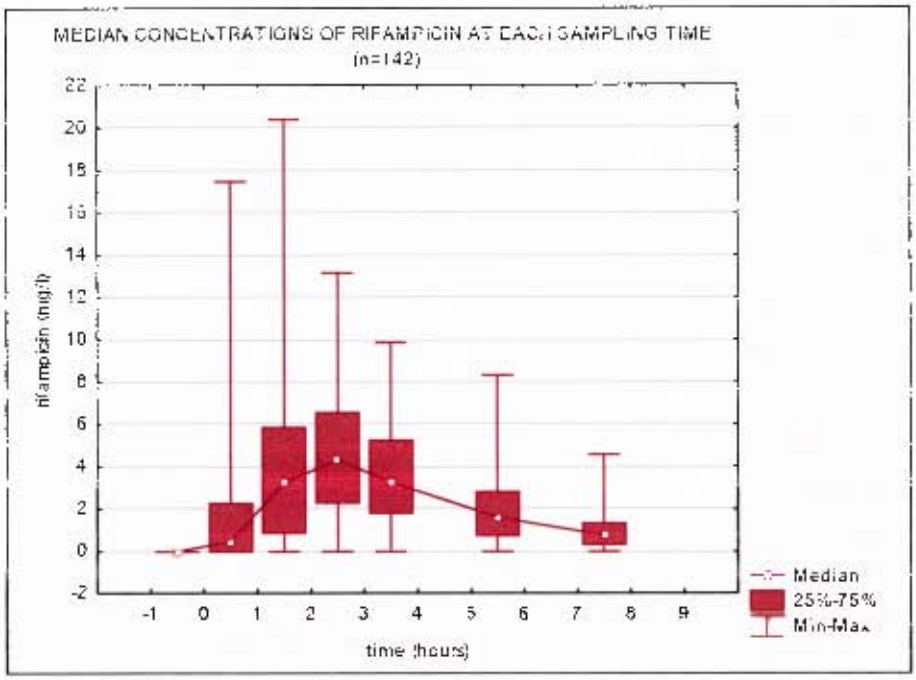
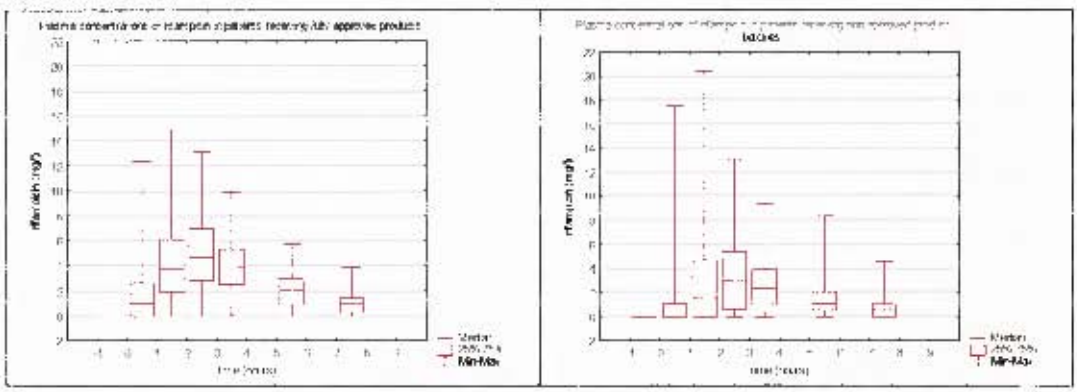


Figure 2.2: The median concentration vs. time curve for rifampicin in patients who received fully approved preparations (graph on left) and in patients who received non-approved drug batches (graph on right).



Correlations between the pharmacokinetic measures were explored using nonparametric methods (table 4).

Clearance and volume of distribution were strongly correlated with each other and had very strong and significant negative correlations with AUC and Cmax. This can be explained by the methods of calculation, which are dependent on the AUCi:

Vz F obs = dose / (λ_z x AUCi)

Cl F obs = dose / AUCi

As the bioavailability cannot be estimated for extravascular drug administration, the absorbed fraction (F) is assigned a value of 1; variability in bioavailability is not adjusted for by this method of calculation. The variables Vz F obs and Cl F obs are not used further as they are unhelpful; the variability in the absorbed fraction being high.

Table 4: Spearman's correlations between the various pharmacokinetic measures of rifampicin expressed by correlation coefficient (p value).

	Lag time							
Tmax	0.5097 (0.0000)	**Tmax**						
Cmax	0.2328 (0.0062)	0.2383 (0.0050)	**Cmax**					
K	-0.0044 (0.9614)	-0.0758 (0.4010)	-0.2350 (0.0058)	**K**				
Half-life	0.0042 (0.9634)	0.0756 (0.4019)	0.2352 (0.0058)	1.0000 (0.0000)	**Half-life**			
AUCt	-0.3052 (0.0003)	-0.2372 (0.0053)	0.9403 (0.0000)	-0.3967 (0.0000)	-0.3977 (0.0000)	**AUCt**		
AUCi	0.2245 (0.0178)	-0.0557 (0.3279)	0.9246 (0.0000)	-0.4605 (0.0000)	0.4608 (0.0000)	0.9899 (0.0000)	**AUCi**	
Vz F obs	0.1719 (0.0653)	0.0827 (0.3593)	-0.9077 (0.0000)	-0.0034 (0.9702)	0.0031 (0.9727)	-0.8669 (0.0000)	0.8210 (0.0000)	**Vz F obs**
Cl F obs	0.1764 (0.0564)	0.0365 (0.6862)	-0.9071 (0.0000)	0.4835 (0.0000)	0.4837 (0.0000)	-0.9658 (0.0000)	-0.9777 (0.0000)	0.8455 (0.0000)

there was strong agreement between peak rifampicin concentrations and the AUC measures.

Although the median lag time was 0; in a substantial minority (46, or 32%) of cases a nonzero lag time (range: 0.44 to 3.06 hours) was recorded. Not surprisingly, the lag time and the time to reach the peak concentration were significantly correlated. These 2 variables showed significant negative correlations with the Cmax and AUCt, and the lag time was also significantly negatively correlated with the AUCi. The relationships between the time to reach the peak concentration, and the Cmax and AUCt, respectively, are shown in figure 3.

A lag time of 0 hours was associated with significantly greater bioavailability than lag times greater than 0 (median Cmax 5.96 mg/l vs. 4.46 mg/l, $p = 0.0007$; median AUCt 22.89 mg.hr.l^{-1} vs. 16.01 mg.hr.l^{-1}, $p = 0.0001$; median AUCi 26.20 mg.hr.l^{-1} vs. 19.20 mg.hr.l^{-1}, $p = 0.0104$). Thus delayed absorption of rifampicin was associated with reduced bioavailability. However, as illustrated in table 5, the effect is significant only for the non-approved product batches. The effect could be related to delayed

dissolution with proportionately greater presystemic metabolism and, or, reduced dissolution or lower drug content in capsules of the non-approved batches.

Figure 3: Plots of the peak rifampicin concentration (A; Spearman's rho = -0.2383, p = 0.0050) and AUCt (B; Spearman's rho = -0.2376, p = 0.0053) vs. the time to reach peak concentration, shows that most patients with delayed peak concentrations had low peak concentrations.

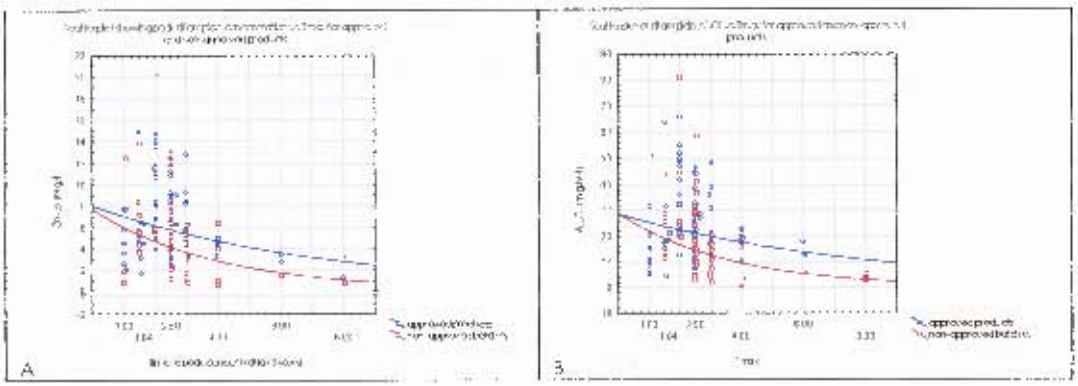


Table 5: The associations between the presence of a lag time, and reduced bioavailability (measured by Cmax, AUCt and AUCi) of rifampicin, using the Kruskal-Wallis test; and the associations between Tmax and Cmax, AuCt and AUCi of rifamycin using Spearman's correlations.

PK measure of bioavailability	All products			Approved batches			Non-approved products		
Kruskal Wallis test	*Median lag = 0*	*Median lag > 0*	*p*	*Median lag = 0*	*Median lag > 0*	*p*	*Median lag = 0*	*Median lag > 0*	*p*
Cmax (mg/l)	3.96 (n=91)	4.46 (n=51)	0.0007	6.26 (n=66)	5.14 (n=23)	0.3797	2.71 (n=26)	2.57 (n=28)	0.0019
AUCt (mg.hr/l)	22.89 (n=90)	16.01 (n=51)	0.0001	22.69 (n=64)	20.32 (n=23)	0.1263	22.37 (n=26)	7.69 (n=28)	0.0009
AUCi (mg.hr/l)	26.05 (n=86)	18.00 (n=39)	0.0104	23.94 (n=62)	19.84 (n=21)	0.7997	21.41 (n=24)	9.87 (n=18)	0.0237
Spearman's correlation with Tmax	*Spearman's rho*		*p*	*Spearman's rho*		*p*	*Spearman's rho*		*p*
Cmax	-0.2303 (n=137)		0.0036	-0.1972 (n=89)		0.2361	-0.3718 (n=48)		0.0081
AUCt	-0.2376 (n=136)		0.0053	-0.1063 (n=87)		0.3270	0.4121 (n=49)		0.0033
AUCi	-0.0882(n=125)		0.3279	0.0274 (n=83)		0.8082	-0.2692 (n=42)		0.0847

The elimination rate constant was significantly negatively correlated with Cmax, and the half-life was significantly positively correlated with Cmax (figure 4). This could reflect saturation of the elimination pathways at higher drug concentrations (an effect described in the literature with increasing doses[89, 92]), or it could indicate that individuals with greater pre-systemic metabolism of the drug also eliminate systemic drug concentrations more rapidly.

The median drug concentrations for each sampling time are shown in table 6. The concentration of antituberculosis agents is often measured at 2 hours after drug dosing; and the Cmax range of 8 – 24 mg/l recommended in the literature is the

same as that of the proposed 2-hour range[6,8]. In this study, the median time to reach the peak concentration (Tmax) was 2.5 hours. The 2.5-hour sampling time was therefore a more reliable indicator of the peak rifampicin concentration in these patients, and this is so, whether they received the approved drug batches, or not (The median rifampicin concentration at 2.5 hours was 4.97 mg/l for the fully approved formulation and 2.87 mg/l for the non-approved batches; p=0.002). The median concentration at 2hrs was 3.56 mg/l (considerably lower than the 4.47 mg/l at 2.5 hours). Those receiving the non-approved batches achieved a median 2-hour concentration of only 2.19 mg/l (95% CI: 0.97 to 3.30), considerably lower than that for the approved products (4.35 mg/l (95% CI: 3.59 to 5.19); p=0.001).

The pre-dose rifampicin concentration was 0 mg/l in all patients indicating that the dose of the day before was eliminated from the systemic circulation within 24 hours.

Figure 4: The association between Cmax, and the half life (A; Spearman's rho = 0.2352, p = 0.0083) and the elimination rate constant (B; Spearman's rho = -0.2350, p = 0.0083).

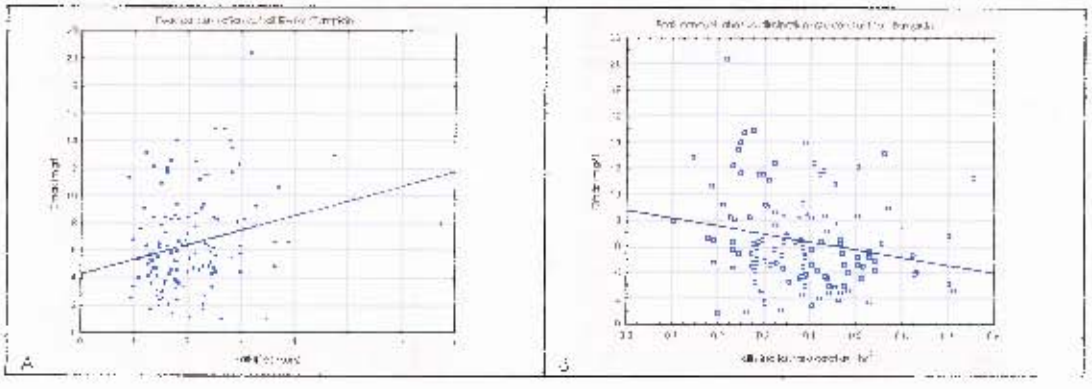


Table 6: Rifampicin concentration at each sampling time, and the correlation of the concentration at each time with the Cmax and AUC[3]. Concentrations below the validated range of the assay assumed a value of 0 mg/l.

Sample time (hr)	N	Median [rifampicin] (mg/l)	Centiles (mg/l)				n when Time = Tmax	Correlation Cmax vs. conc at time[3]	Correlation AUCt vs. conc at time[3]
			0	5th	95th	100th			
0.0	142	0	0.00	0.00	0.00	0.00	-	-	
0.5	142	0	0.00	0.00	3.89	9.89	-	0.296 (0.0004)	0.3526 (0.0003)
1.0	142	.55	0.00	0.00	9.18	17.49	10	0.519 (0.0000)	0.5876 (0.0000)
1.5	142	2.92	0.00	0.00	10.89	19.32	18	0.685 (0.0000)	0.7273 (0.0000)
[illegible]	142	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.5	141	4.47	0.00	0.00	11.76	18.11	69	0.932 (0.0000)	0.9121 (0.0000)
3.0	141	4.29	0.00	0.00	10.55	13.16	21	0.949 (0.0000)	0.9406 (0.0000)
4.0	141	3.31	0.00	0.00	8.02	9.89	11	0.910 (0.0000)	0.9357 (0.0000)
6.0	141	1.62	0.00	0.00	4.88	8.32	3	0.790 (0.0000)	0.8715 (0.0000)
8.0	141	0.76	0.00	0.00	3.32	4.57	4	0.628 (0.0000)	0.7308 (0.0000)

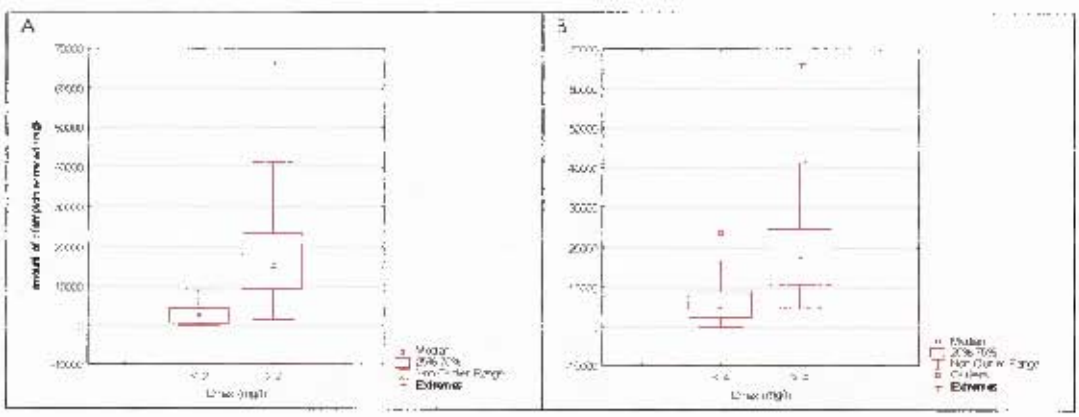
[1] samples lost for 1 subject [2] 1 result invalid [3] spearmans rho (p-value)

Urine collection may provide a non-invasive measure for the assessment of drug exposure. The relationship between the amounts of rifampicin (rifampicin concentration X volume) in the urine and the Cmax and AUCt values were explored. Urine collections were made from 2-4, 4-6, 6-8 and 2-8 hours after drug administration in 104 patients, and from 0 – 8 hours in 10 patients. The volume of urine was recorded and the concentration of rifampicin was determined for each collection. The amount of rifampicin excreted during each time interval (volume x concentration) was then calculated, and the correlations between the amount of rifampicin excreted, and the Cmax and AUCt of rifampicin were calculated. The results are presented in table 7. While good correlations are seen between the amounts excreted (especially data reflecting excretion over a longer period of time) and the pharmacokinetic measures of bioavailability, better correlations were demonstrated for the plasma concentrations at single time points later than 1 hour after drug administration.

Table 7: The correlations between the amount of rifampicin excreted and the pharmacokinetic measures of rifampicin Cmax and AUCt.

Urine collection	n	drug quantity (mg)			Correlation with Cmax (spearman's rho (p-value))	Correlation with AUCt (spearman's rho (p-value))
		Median	Inter-quartile range	Range		
A: 2-4 hour collections	104	5.54	1.41 – 9.19	0 – 31.05	0.6685 (0.000)	0.6470 (0.000)
B: 4-6 hour collections	104	4.34	1.82 – 7.95	0 – 25.37	0.4852 (0.000)	0.5134 (0.000)
C: 6-8 hour collections	104	2.47	0.93 – 4.35	0 – 10.78	0.4768 (0.000)	0.5623 (0.000)
Total **2-8** hr (A+B+C)	104	11.67	6.80 – 19.19	0 – 66.17	0.7077 (0.000)	0.7249 (0.000)
0-8 hour collections	10	24.19	14.22 – 31.76	7.73 – 41.44	0.6606 (0.038)	0.7576 (0.011)
Combined 2-8 and 0-8 hour collections	114	13.04	7.46 – 20.37	0 – 66.17	0.6830 (0.000)	0.7037 (0.000)

Figure 5: Box-and-whisker plots of the amounts of rifampicin excreted in the urine collections (n=114) in patients with very low (Cmax < 2 mg/l ; figure A) and low (Cmax ≥ 4 mg/l; figure B) systemic concentrations of the drug



Urinary collections could provide a practical screening method for identification of patients with low rifampicin concentrations. The figures 5A and B (above) show the urinary excretion of rifampicin in patients with very low (Cmax < 2 mg/l) and low (Cmax < 4 mg/l) plasma drug levels, respectively, compared to those with Cmax concentrations ≥ 2 mg/l (A) and ≥ 4 mg/l (B).

ISONIAZID

The pharmacokinetic measures of isoniazid derived from the individual concentration-time profiles are tabulated below (Table 8).

Table 8: The elimination rate constant (k), half life, lag time, time to reach the peak concentration (Tmax), the peak concentration (Cmax), the area under the concentration-time curve to the last measurable concentration (AUCt), the area under the concentration-time curve to infinity (AUCi), the volume of distribution (Vz F obs) and the clearance (Cl F obs) of isoniazid in each subject, 2 months after admission to Brewelskloof Hospital.

Subject	K (hr^{-1})	half life (hr)	lag time (hr)	Tmax (hr)	Cmax ($mg.l^{-1}$)	AUCt ($mg.l^{-1}.hr$)	AUCi ($mg.l^{-1}.hr$)	Vz F obs (l)	Cl F obs ($l.hr^{-1}$)
1	0.22	3.18	0.0	1.00	9.23	37.09	46.57	29.54	6.44
2	0.20	3.41	0.0	3.00	5.12	21.72	31.20	47.26	9.62
3	0.19	3.59	0.0	2.48	5.47	26.99	36.29	47.84	8.27
4	0.23	3.07	0.0	1.98	6.33	28.63	35.33	37.57	6.49
5	0.26	2.70	0.0	1.00	5.94	23.77	27.87	41.88	10.77
6	0.32	2.17	0.0	1.50	4.71	17.66	19.63	47.91	15.28
7	0.18	3.91	0.0	1.02	8.52	42.27	57.37	22.84	5.20
9	0.22	3.13	0.0	0.50	8.77	37.70	45.03	30.06	6.66
10	0.26	2.70	0.0	2.50	6.71	32.15	37.36	31.26	8.03
11	0.29	2.40	0.0	1.52	7.17	26.04	30.47	34.11	9.85
13	0.30	2.34	0.0	2.50	5.67	23.17	27.41	37.01	10.94
14	0.25	2.78	0.0	1.70	5.10	22.13	28.27	45.70	11.41
15	0.26	2.71	0.0	1.50	10.37	37.97	51.05	22.99	5.88
16	0.25	2.76	0.0	1.00	9.34	36.86	49.91	26.02	6.53
17	0.35	2.00	0.0	0.50	13.23	55.83	60.16	14.31	4.96
18	0.40	1.75	0.0	1.00	7.42	33.88	36.57	20.70	8.20
19	0.17	4.06	0.0	1.00	11.49	49.11	70.48	24.55	4.26
20	0.19	3.64	0.0	0.50	13.89	51.32	57.91	23.17	4.42
22	0.23	2.99	0.0	2.00	11.50	29.39	42.34	30.56	7.08
23	0.30	2.29	0.0	3.00	6.89	30.99	35.35	27.98	8.49
24	0.17	4.05	0.0	1.48	12.12	50.39	68.39	25.66	4.39
25	0.29	2.40	0.0	1.02	11.75	44.78	51.50	20.20	5.83
26	0.13	5.17	0.0	1.50	6.86	30.92	53.04	42.22	5.66
27	0.27	2.60	0.0	1.03	7.72	36.34	43.79	25.25	6.86
29	0.66	1.05	0.0	2.52	7.35	24.36	24.74	18.29	15.13
30	0.50	1.40	0.0	1.00	10.52	27.27	29.34	20.64	10.22
31	0.15	4.55	0.0	2.03	7.41	28.33	38.44	51.21	7.80
34	0.25	2.73	0.0	1.03	8.14	32.75	38.71	30.51	7.75
37	0.16	4.36	0.0	1.98	7.64	25.53	34.38	55.07	8.73
41	0.13	5.29	0.0	0.98	7.32	26.49	40.17	57.01	7.47
42	0.10	7.15	0.0	2.50	4.52	22.42	45.89	67.47	6.54
43	0.21	3.30	0.0	1.08	4.42	19.65	23.88	59.87	12.56
44	0.18	3.84	0.0	1.50	9.20	42.47	56.46	29.41	5.31
45	0.50	1.37	0.0	0.95	6.82	17.10	17.98	33.10	16.69
46	0.25	2.73	0.0	3.00	4.38	20.99	25.85	45.72	1.61
47	0.28	2.49	0.0	2.00	5.54	7.06	19.64	54.61	15.27

Table 8 continued

	Ke (hr-1)	half life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/L)	AUC1 (mg/L*hr)	AUC∞ (mg/L*hr)	Vz/F obs (L)	CL/F obs (L/hr)
48	0.25	2.82	0.0	2.00	4.12	16.19	19.93	61.51	15.05
49	0.38	1.81	0.0	2.03	3.16	11.92	12.84	61.13	23.37
50	0.27	2.55	0.0	0.50	15.04	35.79	39.60	27.89	7.58
51	0.36	1.91	0.0	0.50	5.39	11.08	15.11	54.70	19.86
52	0.09	7.39	0.0	1.50	2.29	7.14	9.20	112.31	32.62
53	0.48	1.46	0.0	3.00	0.99	2.77	18.96	66.47	31.64
54	0.37	1.87	0.0	2.02	3.39	20.70	24.97	32.50	12.02
55	0.38	1.83	0.0	2.52	7.95	24.90	26.88	29.49	11.16
56	0.22	3.12	0.0	0.50	9.20	39.04	46.52	29.06	6.45
57	0.50	1.39	0.0	1.50	5.97	22.05	23.00	26.06	13.04
58	0.28	2.46	0.0	4.02	6.93	33.19	41.25	25.62	7.27
59	0.24	2.88	0.0	1.48	9.43	38.47	43.14	33.45	8.66
60	0.19	3.59	0.0	2.50	5.87	31.87	44.06	35.25	6.81
61	0.37	1.85	0.0	1.00	4.55	17.12	18.19	44.03	16.50
63	0.37	1.87	0.0	0.50	6.18	17.84	18.94	42.82	15.84
64	0.25	2.82	0.0	0.50	7.55	24.96	30.07	40.52	9.98
65	0.21	3.28	0.0	0.50	10.26	32.26	34.25	27.27	8.76
66	0.33	2.08	0.0	0.50	4.53	16.01	17.41	51.82	17.23
67	0.34	2.04	0.0	2.03	4.40	16.03	17.73	49.69	16.22
68	0.37	1.87	0.0	2.71	4.66	19.80	21.45	37.72	13.99
70	0.19	3.64	0.0	1.00	10.56	48.47	64.95	24.32	4.63
71	0.25	2.78	0.0	2.52	5.91	23.15	28.97	27.70	6.90
72	0.28	2.51	0.0	0.50	10.27	37.49	42.55	25.54	7.04
73	0.27	2.56	0.0	0.50	8.00	27.03	31.39	35.28	9.56
74	0.22	3.18	0.0	1.50	9.40	40.88	53.22	26.84	5.86
75	0.29	2.37	0.0	1.00	8.86	30.72	35.12	29.16	8.54
77	0.27	2.59	0.0	0.50	9.78	35.69	40.72	27.57	7.37
80	0.20	3.43	0.0	2.50	4.95	20.21	26.44	56.19	11.35
81	0.17	4.12	0.0	0.98	5.36	24.67	34.28	52.07	8.75
83	0.16	4.26	0.0	0.50	5.34	16.77	22.43	82.12	13.39
84	0.17	4.09	0.0	1.50	5.10	22.95	31.16	85.96	9.63
86	0.24	2.85	0.0	1.02	6.85	27.04	32.84	37.55	9.13
87	0.12	5.58	0.0	3.53	0.49	1.74	3.49	740.31b	85.85b
88	0.25	2.79	0.0	1.50	7.46	24.56	31.23	38.67	9.61
89	0.16	4.24	0.0	3.00	4.48	25.31	37.42	49.03	8.02
90	0.13	5.24	0.0	1.50	4.50	23.53	36.73	61.76	8.17
91	0.15	4.63	0.0	2.50	5.02	39.51	64.76	30.95	4.63
92	0.45	1.56	0.0	1.50	4.74	11.59	13.23	60.92	22.68
93	0.18	3.91	0.0	2.52	6.93	42.15	58.72	28.80	5.11
94	0.26	2.70	0.0	2.00	6.05	26.12	35.32	33.06	8.49
95	0.23	3.05	0.0	0.50	10.09	37.53	46.28	28.52	6.48
96	0.15	4.64	0.0	1.06	5.44	27.85	32.51	60.80	9.23
97	0.28	2.44	0.0	2.00	8.31	26.62	32.56	32.44	9.21
101	0.22	3.15	0.0	0.50	11.35	38.45	46.54	29.27	6.45
103	0.23	3.02	0.0	0.55	6.26	27.54	35.38	39.21	8.99

Tab e 8 continuen

	[ml/l]	[hr]	[hr]	[hr]	[mg/l]	[mg · hr]	[mg/l · hr]	[l]	[hr]
104	0.42	1.64	0.6	2.50	10.61	32.94	35.78	19.81	8.39
105	0.67	1.11	0.5	3.00	8.93	24.39	24.94	19.31	12.09
106	0.20	3.42	0.0	4.02	5.59	30.15	42.49	34.97	7.06
107	0.27	2.56	0.0	1.50	9.30	40.6	47.2	29.52	6.37
108	0.50	1.37	0.0	2.50	8.66	31.09	32.51	18.23	9.20
109	0.65	1.07	0.0	1.50	8.08	20.36	20.73	22.67	14.47
110	0.30	2.94	0.0	2.53	7.11	37.20	39.44	49.07	14.52
111	0.52	1.35	0.0	3.00	7.47	23.53	24.74	23.55	12.73
112	0.29	2.42	0.0	2.50	8.06	35.08	40.49	25.89	7.41
113	0.41	1.77	0.0	1.50	9.77	40.22	42.54	17.38	7.05
115	0.38	1.82	0.0	1.52	6.21	23.03	24.87	31.69	13.06
117	0.34	2.04	0.0	2.03	8.90	33.40	37.64	23.66	8.06
118	0.25	2.83	0.0	1.98	9.29	47.06	51.27	29.87	5.86
119	0.50	1.39	0.0	1.02	6.01	19.87	20.49	29.44	14.64
120	*	*	0.0	2.02	11.27	*	*	*	*
121	0.29	2.40	0.0	2.50	10.64	46.96	56.46	18.37	5.31
122	0.39	1.80	0.5	2.50	5.17	17.56	20.24	32.39	14.82
123	0.23	3.00	0.5	3.00	9.59	35.93	46.60	27.86	6.44
124	0.37	1.90	1.6	3.02	10.40	23.35	26.45	31.03	11.34
125	0.36	1.81	0.0	2.55	3.84	15.77	16.93	46.30	17.72
126	0.32	2.15	0.5	1.05	12.85	52.41	57.88	16.09	5.19
127	0.33	2.13	0.5	4.02	7.91	32.29	38.64	23.74	7.27
128	0.44	1.56	0.0	1.53	9.14	20.44	21.59	31.33	13.89
130	0.29	2.46	0.0	0.50	5.30	19.88	22.56	47.14	13.30
131	0.19	3.59	0.5	1.96	3.99	18.56	25.48	60.95	21.78
132	0.14	4.81	0.0	3.00	4.64	29.16	36.82	56.59	8.15
133	0.23	2.98	0.0	1.50	5.86	22.15	28.01	68.97	15.07
134	0.26	2.63	0.0	2.00	7.59	30.79	37.44	31.56	8.01
135	0.15	4.33	0.0	3.03	4.47	20.11	32.05	51.11	9.99
136	0.19	3.73	0.0	1.02	4.39	18.27	23.72	68.01	12.65
137	0.24	2.88	0.0	2.57	9.76	12.96	15.98	78.09	18.77
138	0.05	8.41	0.0	2.00	4.07	14.93	24.62	147.89	12.19
139	0.41	1.71	0.0	1.00	6.58	27.82	29.42	25.13	10.20
141	0.21	3.33	0.0	2.00	7.62	31.06	40.00	36.26	7.50
142	0.19	3.58	0.0	1.03	14.13	64.02	87.15	17.79	3.44
143	0.12	5.93	0.0	3.00	9.31	26.47	50.43	30.92	5.95
144	0.21	3.36	0.0	1.64	7.61	29.27	42.64	34.07	7.04
145	0.18	3.80	0.0	1.00	5.21	23.54	32.29	50.86	9.29
46	0.19	3.62	0.0	2.53	3.97	25.50	34.97	55.64	6.58
147	0.20	3.46	0.0	1.58	6.94	30.52	40.41	37.03	7.42
148	0.18	3.85	0.0	1.05	8.57	31.34	42.68	39.25	7.06
149	0.30	2.32	0.0	2.03	3.94	18.19	21.58	44.60	13.90
150	0.25	2.82	0.0	0.50	5.39	21.21	25.02	48.79	11.99
151	0.20	3.50	0.0	1.00	7.94	29.40	38.84	39.06	7.12
152	0.24	2.91	0.0	0.50	9.22	19.27	24.63	57.20	12.18

Table 8 continued

	K (hr⁻¹)	half-life (hr)	lag time (hr)	tmax (hr)	Cmax (mg.l⁻¹)	AUC1 (mg.l⁻¹.hr)	AUCi (mg.l⁻¹.hr)	V2F obs (l)	CLF obs (l.hr⁻¹)
153	0.23	3.05	.0	3.00	4.78	14.63	21.47	67.49	13.92
154	0.53	1.32	0.0	0.56	4.42	5.61	9.38	60.76	31.97
155	0.25	2.82	0.0	1.50	3.69	14.85	17.86	68.36	16.80
156	0.09	7.98	0.0	2.50	4.19	17.95	34.09	101.36	8.80
158	0.48	1.44	0.0	0.50	5.15	12.60	13.77	46.39	21.79
159	0.17	4.20	0.0	2.50	4.75	25.00	38.21	47.56	7.85
160	0.27	1.53	0.0	3.00	3.62	12.03	12.93	34.71	22.39
161	0.17	4.09	0.0	0.50	6.56	17.05	21.40	82.78	14.02
162	0.26	2.65	0.0	1.02	5.78	23.48	27.55	47.63	10.89
163	0.21	3.33	0.0	1.50	5.93	25.22	32.10	44.84	9.35
164	0.32	2.19	0.0	0.53	6.58	13.02	15.02	63.08	19.98
165	0.23	3.04	0.0	1.53	5.23	20.86	26.82	45.10	11.17
166	0.23	3.02	0.5	2.52	3.41	14.21	17.71	73.76	16.94
167	0.69	1.01	0.0	1.50	3.11	6.21	7.24	60.44	41.44
168	0.25	2.76	0.0	1.50	4.59	16.16	18.97	62.81	15.81
169	0.29	2.38	0.0	1.00	4.03	12.58	15.75	65.44	19.05

Data summary

	K (hr⁻¹)	half-life (hr)	lag time (hr)	tmax (hr)	Cmax (mg.l⁻¹)	AUC1 (mg.l⁻¹.hr)	AUCi (mg.l⁻¹.hr)	V2F obs (l)	CLF obs (l.hr⁻¹)
n	141	141	142	142	142	141	141	141	141
median	0.25	2.76	0.0	1.5	6.47	24.96	32.51	37.55	9.21
5th centile	0.13	1.35	0.0	0.5	3.55	11.62	13.29	18.46	4.98
95th centile	0.51	5.23	0.5	3.0	12.07	48.32	60.27	77.65	23.90

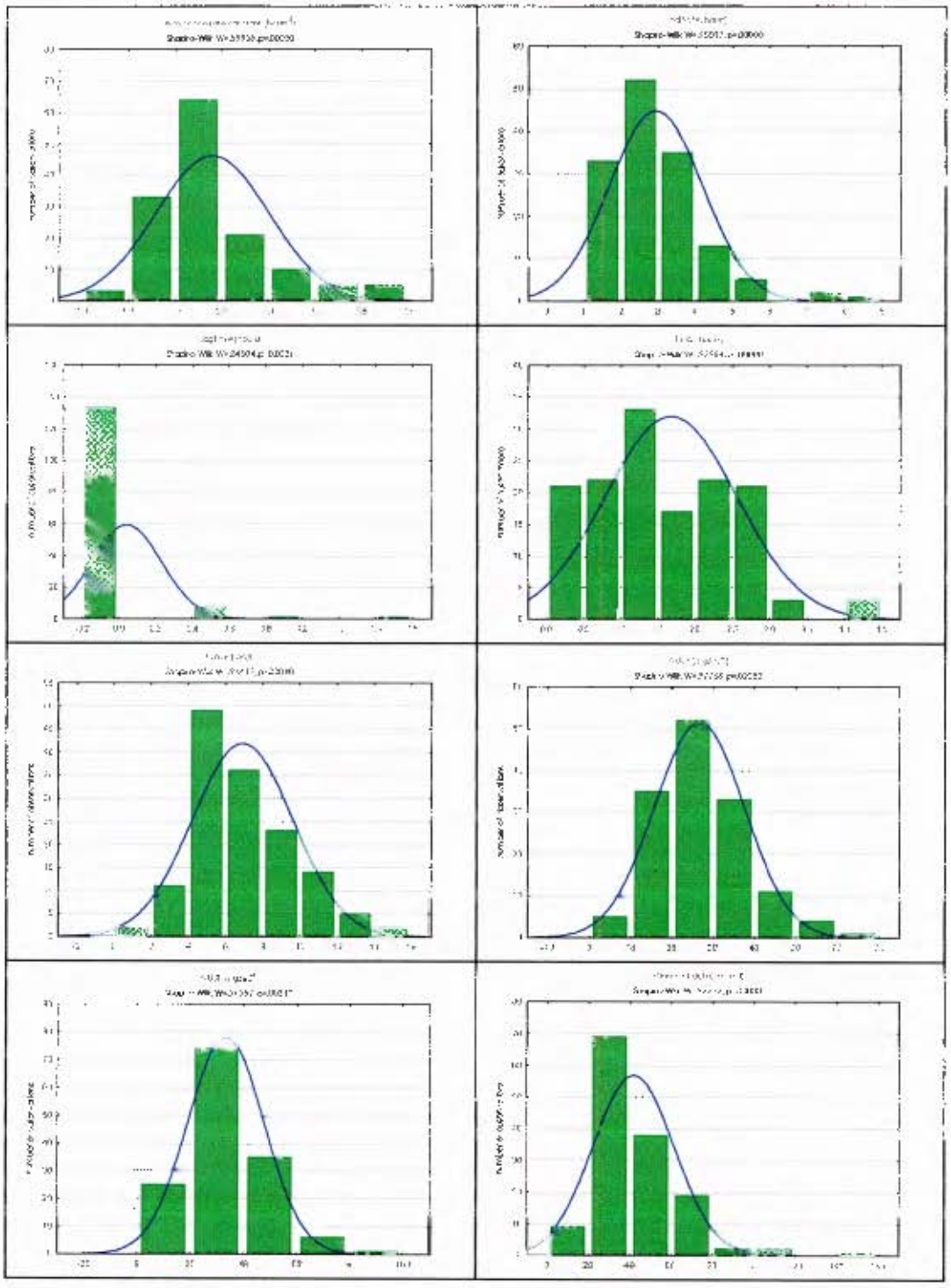
* Data excluded from further analysis as sampling was only available until 3 hours after drug administration.
# Outlying values due to very low AUC; excluded from summary statistics.

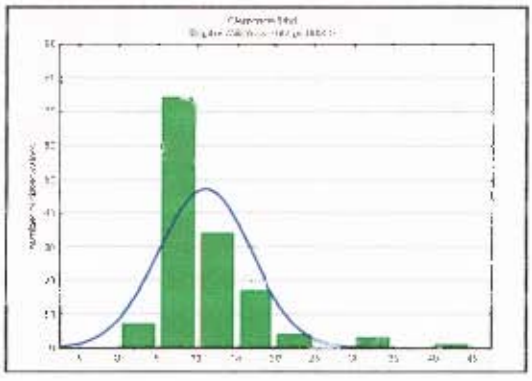
Peak isoniazid concentrations were detected between 0 and 3.0 hours in 124 of 142 cases. Half-hourly blood sampling during this period allowed reasonably accurate determination of the peak rifampicin concentration.

In only 3 (2 %) of the cases was the ratio of AUCi to AUCi less than 0.80. The sampling schedule was, therefore, adequate for detecting the overall drug exposure in almost all patients.

The distribution of each pharmacokinetic measure is illustrated in figure 6. The Shapiro-Wilk test for normality showed that all the pharmacokinetic measures were skewed.

Figure 6: Histograms for each pharmacokinetic measure of lomefloxacin show the distribution of the data in 142 patients. One outlying observation (subject 87) was excluded for volume of distribution and clearance.





The median Cmax value for isoniazid was 6.47 mg/l (95% CI of median: 5.90 – 7.06), with the 5th – 95th centile range of 3.55 – 12.07 mg/l. Isoniazid concentrations were therefore within and greater than the 3 - 5 mg/l recommended range[56,66,67,82].

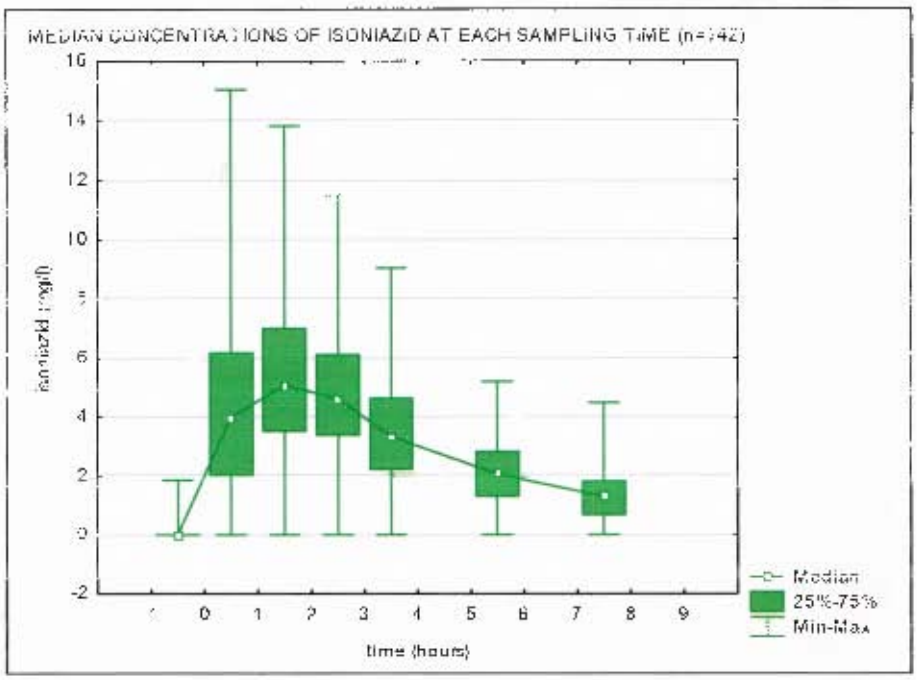
The median time to reach the peak concentration was 1.5 hours (5th – 95th centile range: 0.5 – 3 hours). Only 9 patients had measurable lag times for isoniazid and these patients did not have lower peak concentrations of isoniazid (median Cmax for those with delayed isoniazid absorption: 7.91 mg/l; 95% CI: 4.05 – 10.52 vs. 6.39 mg/l; 95% CI: 5.90 - 6.99 for those with no measurable delay; p=0.7535). Seven of the 9 subjects with delayed onset of absorption received a fixed dose combination of isoniazid and rifampicin on the day of pharmacokinetic sampling and all patients with delayed onset of isoniazid absorption, also had delayed (i.e. a nonzero lag time) rifampicin absorption.

The median AUC$_t$ was 24.96 mg.hr.l^{-1} (95% CI: 23.23 – 27.01), and that of the AUC$_i$ 32.51 mg.hr.l^{-1} (95% CI: 29.30 – 35.33). The median half-life was 2.76 hours (95% CI: 2.57 - 2.90), and the elimination rate constant had a median value of 0.25 hr^{-1} (95% CI: 0.24 - 0.27).

No obvious bimodality was observed in the distribution of the half-life, the elimination rates, the peak concentrations or the AUC (figure 6). The di- or tri-chotomization conferred by acetylator status[125] was therefore obscured; possibly by other factors contributing significantly to pharmacokinetic variability or because of patient factors making the phenotypic differences less well defined[66,124]. Covariate factors influencing the pharmacokinetic measures are explored in the next chapter.

The median concentrations of isoniazid at each time point are represented in figure 7.

Figure 7: The median concentration vs. time curve for isoniazid. Error bars indicate the ranges of concentrations at each sampling time and the boxes represent the 25% - 75% percentile ranges.



The isoniazid concentrations at each time point are summarized in table 9, below. Most patients had eliminated the previous day's dose prior to blood sampling.

Blood sampling at 2-hours yields concentrations reasonably correlated with the peak concentration (Spearman's rho: 0.792).

Table 9: Isoniazid concentration at each blood sampling time. Concentrations below the validated range of the assay assumed a value of 0 mg/L.

Time of sampling (hr)	N	Median concentration (mg/l)	Centiles (mg/l)				Time-Tmax n	Correlation Cmax vs. conc at time[3]
			0	5th	95th	100th		
0.0	142	0.00	0.00	0.00	0.92	1.85	0	-
0.5	142	3.17	0.00	0.00	10.19	15.04	27	0.513(0.0000)
1.0	142	4.45	0.00	0.41	10.56	14.13	27	0.682(0.0000)
1.5	142	5.10	0.00	1.29	10.30	13.84	28	0.750(0.0000)
2.0	142	4.96	0.35	1.82	9.77	13.64	19	0.792(0.0000)
2.5	142	4.79	0.00	1.69	9.43	11.50	23	0.786(0.0000)
3.0	142	4.40	0.00	1.58	9.96	10.60	15	0.735(0.0000)
4.0	142	3.35	0.00	1.05	6.84	7.04	3	0.658(0.0000)
6.0	142	2.06	0.00	0.45	4.10	5.18	0	0.533(0.0000)
8.0	140[1,2]	1.31	0.00	0.00	2.96	4.48	0	0.425(0.0000)

[1] samples lost for 1 subject
[2] result invalid
[3] spearmans rho (p-value)

Correlations between the pharmacokinetic measures were explored using nonparametric methods (table 10).

Table 10: Spearman's correlations between the various pharmacokinetic measures of isoniazid expressed as correlation coefficient (p-value).

	Lag time							
Tmax	0.3038 (0.0002)	**Tmax**						
Cmax	0.0031 (0.9690)	-0.2776 (0.0010)	**Cmax**					
K	0.1127 (0.1851)	0.0199 (0.8147)	0.0319 (0.7074)	**K**				
Half-life	-0.1127 (0.1833)	-0.0195 (0.8183)	-0.0327 (0.7059)	1.0000 (0.0000)	**Half-life**			
AUCt	0.0628 (0.4594)	-0.1148 (0.1752)	0.8607 (0.0000)	-0.2162 (0.0094)	0.2162 (0.0094)	**AUCt**		
AUCi	0.0811 (0.3391)	0.0378 (0.6564)	0.7361 (0.0000)	-0.4595 (0.0000)	0.4595 (0.0000)	0.9436 (0.0000)	**AUCi**	
Vz F obs	-0.0480 (0.5715)	0.0379 (0.6557)	0.8229 (0.0000)	0.3634 (0.0000)	0.3635 (0.0000)	0.7796 (0.0000)	0.5897 (0.0000)	**Vz F obs**
Cl F obs	0.0783 (0.3560)	0.0313 (0.7179)	-0.7311 (0.0000)	0.4550 (0.5000)	-0.4549 (0.0000)	-0.9362 (0.0000)	-0.9905 (0.0000)	0.6061 (0.0000)

A lag-time was detected in a small minority of cases; and a lag-time was significantly associated with a delayed peak concentration.

Delayed absorption was associated with lower peak concentrations as evidenced by the significant but weak association between Tmax and Cmax. In contrast, an association was not reflected between Tmax and AUCt or AUCi.

The peak isoniazid concentration was not associated with the elimination rate constant or the half-life, indicating independence of the latter 2 parameters, as is consistent with first order elimination.

As expected, Cmax was strongly and significantly correlated with AUCt and AUCi. The half-life and elimination rate constant were also significantly associated with the AUC values, the AUCi in particular.

Clearance and volume of distribution had strong and significant negative correlations with AUC and Cmax. For the reasons cited in the section on rifampicin (above), the variables Vz F obs and Cl F obs are not used further.

PYRAZINAMIDE

The pharmacokinetic measures of pyrazinamide derived from the individual concentration-time profiles are tabulated below (Table 11).

Table 11: the elimination rate constant (K), half-life, lag time, time to reach the peak concentration (Tmax), the peak concentration (Cmax), the area under the concentration-time curve to the last measurable concentration (AUCt), the area under the concentration-time curve to infinity (AUCi), the volume of distribution (Vz F obs) and the clearance (Cl F obs) of pyrazinamide for each subject, 2 months after admission to Immwesiso Hospital.

Subject	K (hr⁻¹)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg.l⁻¹)	AUCt (mg.l⁻¹.hr)	AUCi (mg.l⁻¹.hr)	Vz F obs (l)	Cl F obs (l.hr⁻¹)
1	0.12	6.02	0.0	1.52	75.25	358.01	652.94	19.65	2.26
2	0.23	3.08	0.0	6.00	45.68	234.10	316.70	21.04	4.71
3	0.16	4.25	0.0	1.98	48.87	256.72	379.74	24.25	3.95
4	0.17	4.18	0.0	1.50	46.38	242.47	351.59	17.15	2.84
5	0.13	5.31	0.0	1.00	58.79	318.68	515.20	29.54	3.88
6	0.12	5.99	0.0	1.50	46.51	262.88	436.05	39.45	4.57
7	0.15	4.74	0.0	1.50	50.57	238.75	360.35	28.50	5.46
9	0.12	5.89	0.0	1.00	52.37	293.42	499.68	25.50	3.00
10	0.10	7.11	0.0	2.50	53.51	306.71	625.49	24.58	2.40
11	0.12	5.93	0.0	1.52	45.52	253.72	449.58	28.56	3.34
13	0.11	6.20	0.0	2.50	43.70	244.14	439.80	30.52	3.41
14	0.12	7.30	0.0	1.70	47.66	264.19	499.52	40.52	4.00
15	0.08	8.20	0.0	1.50	79.13	466.59	569.39	18.30	1.55
16	0.18	3.87	0.0	1.00	42.98	275.84	275.69	30.42	5.44
17	0.08	8.23	0.0	1.00	65.35	387.38	798.55	22.31	1.88
18	0.16	4.27	0.0	2.02	41.14	231.83	345.23	26.46	4.34
19	0.14	4.93	0.0	1.50	51.10	278.57	435.16	24.53	3.45
20	0.11	6.23	0.0	0.50	69.61	407.69	674.59	19.98	2.22
27	0.12	5.73	0.0	2.02	54.39	319.80	546.20	30.25	3.51
23	0.06	11.61	0.0	2.50	61.24	334.77	1102.11	30.40	1.81
24	0.11	6.10	0.0	1.48	57.20	323.94	574.13	22.99	2.61
25	0.12	5.97	0.0	1.02	69.80	419.24	724.27	17.85	2.07
26	0.07	9.62	0.0	2.00	63.36	378.52	945.92	27.34	2.11
27	0.17	4.13	0.0	2.00	50.91	280.22	375.56	23.82	5.99
29	0.14	5.07	0.0	2.00	70.96	400.57	654.52	16.77	2.29
30	0.16	4.34	0.0	1.50	58.51	294.22	476.90	22.02	3.51
31	0.08	8.84	0.0	1.50	74.57	484.91	1066.85	17.92	1.41
34	0.13	5.17	0.0	1.50	53.46	287.82	457.91	24.45	3.28
37	0.11	6.41	0.0	1.98	61.87	373.67	677.95	20.30	2.17
41	0.12	5.96	0.0	1.05	57.94	280.57	470.71	36.53	4.25
42	0.10	7.15	0.0	3.00	40.41	245.45	508.04	9.14	0.89
43	0.10	6.73	0.0	2.05	37.59	230.24	421.73	10.37	1.04
44	0.14	5.03	0.0	1.03	54.48	299.92	466.92	7.00	0.96
45	0.19	3.59	0.0	1.50	46.54	282.42	510.66	7.63	1.47
46	0.07	9.49	0.0	3.00	49.89	335.27	820.88	7.50	0.55
47	0.09	7.75	0.0	2.50	36.44	229.37	482.24	10.43	0.93

Table 11 continued

	Ke (hr⁻¹)	t½ Elim (hr)	Lag Time (hr)	Tmax (hr)	Cmax (mcg/ml)	AUC1 (mg...hr)	AUC1 (mcg/...hr)	Vd Obs (l)	CL Obs (l/hr)
48	0.07	10.18	0.0	2.00	63.40	395.03	985.32	29.82	2.03
49	0.11	6.48	0.0	2.03	41.35	239.43	447.21	41.79	4.47
50	0.10	7.22	0.0	0.50	91.84	453.17	852.48	18.34	1.76
51	0.09	8.02	0.0	0.50	59.18	345.83	706.54	32.75	2.83
52	0.05	12.89	0.0	1.50	40.28	262.78	790.70	35.27	1.90
53	0.06	10.85	0.0	2.00	28.00	166.54	427.07	73.31	4.68
54	0.20	3.40	0.0	2.52	56.50	224.73	359.56	20.49	4.17
55	0.11	6.56	0.0	2.02	50.65	209.22	390.87	36.34	3.84
56	0.12	5.67	0.0	0.50	73.50	413.62	684.14	17.93	2.19
57	0.10	6.82	0.0	1.50	52.67	247.08	414.25	35.61	3.62
58	0.13	5.38	0.0	4.02	61.39	379.64	666.36	17.51	2.25
59	0.09	8.90	0.0	2.50	60.26	346.76	785.49	24.66	1.92
60	0.11	6.57	0.0	2.50	52.72	302.21	592.86	23.97	2.53
61	0.10	7.10	0.0	.50	30.95	184.66	350.50	43.85	4.28
63	0.09	7.30	0.0	1.98	51.13	315.69	507.90	34.67	3.29
64	0.11	6.47	0.0	3.50	60.60	308.98	547.00	34.15	3.66
65	0.13	5.30	0.0	0.50	81.93	336.11	514.97	22.20	2.71
66	0.10	7.03	0.0	2.02	56.32	345.55	666.39	30.37	3.50
67	0.12	5.93	0.0	2.03	48.39	293.37	518.06	24.75	2.90
68	0.12	5.88	0.0	2.7	50.59	301.99	527.64	23.66	2.79
70	0.15	4.75	0.0	.50	63.02	277.96	429.79	23.92	3.49
71	0.14	4.86	0.0	4.03	22.93	131.59	227.58	31.50	4.49
72	0.14	5.14	0.0	0.50	31.65	138.63	209.35	35.39	4.78
73	0.17	4.14	0.0	1.00	49.04	254.93	363.51	24.64	4.13
74	0.11	6.49	0.0	2.52	51.14	287.52	526.52	26.55	2.85
75	0.15	4.65	0.0	1.00	55.28	292.56	447.09	22.53	3.36
77	0.11	6.11	0.0	1.00	70.98	368.11	622.12	28.28	3.21
80	0.09	7.44	0.0	2.50	55.50	265.42	561.27	28.70	2.67
81	0.11	6.42	0.0	98	49.06	291.41	519.45	26.74	2.89
83	0.17	4.16	0.0	1.00	56.23	265.67	371.65	32.32	5.38
84	0.08	8.33	0.0	1.50	51.14	320.13	675.54	26.69	2.22
86	0.15	4.71	0.0	2.02	56.43	310.25	468.74	21.73	3.20
87	0.09	8.10	0.0	0.53	1.47	9.02	18.69	937.79	80.291
88	0.08	8.39	0.0	1.00	78.61	432.88	900.65	20.16	1.67
89	0.12	5.59	0.0	3.00	50.66	309.18	541.37	22.35	2.77
90	0.08	8.41	0.0	2.00	54.59	386.32	897.10	28.98	2.39
91	0.09	7.49	0.0	2.50	71.79	345.17	743.77	21.78	2.02
92	0.16	4.24	0.0	1.00	51.30	73.08	294.71	41.52	6.79
93	0.07	10.36	0.0	1.50	81.57	481.49	1246.76	17.99	1.20
94	0.12	5.78	0.0	1.50	70.19	396.83	677.48	18.47	2.21
95	0.13	5.33	0.0	1.00	65.03	310.19	490.00	23.52	3.06
96	0.14	4.98	0.0	2.00	40.50	200.25	307.67	46.77	6.50
97	0.06	12.40	0.0	2.50	60.81	326.43	940.82	28.51	1.59
101	0.14	5.02	0.0	0.50	77.33	323.12	509.53	28.42	3.93
103	0.10	7.20	0.0	2.00	46.55	274.66	540.77	38.40	3.70

Table 11 continued

	λz (hr⁻¹)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg·L⁻¹)	AUC (mg·L⁻¹·hr)	AUC (mg·L⁻¹·hr)	Vz F obs (L)	Cl F obs (L·hr⁻¹)
104	0.13	5.41	0.0	2.50	46.34	269.33	455.34	25.73	3.29
105	0.14	5.05	0.0	1.52	52.94	259.06	407.14	24.83	3.68
106	0.17	4.17	0.0	3.02	47.88	256.58	395.20	22.84	3.80
107	0.14	4.98	0.0	1.00	65.58	349.67	480.32	22.44	3.12
108	0.14	4.91	0.0	0.55	54.20	279.66	418.12	25.40	3.59
109	0.12	5.93	0.0	2.00	52.23	295.36	523.57	24.51	2.86
110	0.15	4.33	0.0	1.50	54.99	300.10	479.57	21.81	3.47
111	0.13	5.22	0.0	1.50	46.97	254.44	420.17	26.86	3.57
112	0.10	7.28	0.0	2.50	45.25	272.55	536.68	39.15	3.73
113	0.11	6.34	0.0	1.50	59.88	329.06	615.55	29.71	3.25
115	0.11	6.17	0.0	2.02	51.12	317.30	567.84	23.50	2.64
117	0.14	5.10	0.0	1.50	54.14	289.65	450.97	26.09	3.33
118	0.13	5.32	0.0	1.98	44.90	245.83	402.50	28.61	3.73
119	0.13	5.20	0.0	1.50	59.70	322.30	501.99	29.87	3.98
120	=	=	0.0	2.02	52.50	=	=	=	=
121	0.17	4.14	0.0	1.02	58.22	339.92	49 .89	18.57	3.11
122	0.11	6.14	0.0	4.05	53.23	311.21	611.17	21.73	2.43
123	0.17	4.08	0.0	2.08	51.11	266.36	372.52	23.70	4.03
124	0.11	6.04	0.0	4.05	57.87	321.98	639.14	20.45	2.35
125	0.12	5.97	0.0	2.06	50.80	288.41	501.60	25.75	2.99
126	0.14	5.07	0.0	1.05	65.36	352.90	537.14	20.43	2.79
127	0.12	5.96	0.0	2.53	64.03	354.52	662.3	19.47	2.76
128	0.14	4.92	0.0	1.53	6 .50	342.08	529.64	26.53	3.78
130	0.07	9.45	0.0	0.52	35.98	219.31	504.09	41.26	2.96
131	0.17	3.97	0.0	3.05	35.49	212.93	309.68	27.74	4.84
132	0.13	4. 9	0.0	1.50	37.39	20 .51	275.88	32.90	5.44
133	0.2	3.27	0.0	2.52	34.57	182.56	253.90	37.15	7.88
134	0.16	4.24	0.0	5.03	29.22	179.17	241.57	23.22	5.69
135	0.13	5.36	0.0	3.03	43.69	227.79	414.37	27.98	3.47
136	0.09	7.67	0.0	2.50	44.8	276.76	564.89	39.18	3.54
137	0.11	6.59	0.0	2.52	36.64	224.09	437.5	32.62	3.43
138	0.11	6.31	0.0	2.52	47.84	287.69	521.25	34.94	3.84
139	0.14	4.94	0.0	1.09	57.21	296.65	454.32	23.51	3.30
141	0.14	5.17	0.0	2.03	63.82	325.97	521.52	28.28	3.83
142	0.11	6.19	0.0	2.00	44.53	245.64	458.36	29.23	3.27
143	0.13	5.38	0.0	3.00	32.57	189.03	319.62	24.29	3.13
144	0.17	4.04	0.0	1.11	41.71	214.95	298.01	19.54	3.36
145	0.07	9.60	0.0	1.00	65.52	385.34	907.75	22.88	1.60
146	0.17	4.20	0.0	2.53	70.60	432.77	676.64	17.91	2.96
147	0.09	8.06	0.0	2.16	86.57	510.23	1087.49	6.03	1.38
148	0.12	5.75	0.0	1.05	69.27	349.50	589.08	21.13	2.55
149	0.09	7.71	0.0	4.06	46.16	284.29	645.50	25.86	2.32
150	0.15	4.71	0.0	1.50	56.17	265.06	408.40	33.28	4.90
151	0.07	9.75	0.0	1.00	45.12	257.64	580.48	36.36	2.58
152	0.13	5.31	0.0	2.00	44.83	248.45	405.27	28.34	3.70

Table 11 continued

	K (hr⁻¹)	half-life (hr)	tlag *min (hr)	tmax (hr)	Cmax (mg/L)	AUCt (mg/L*hr)	AUCi (mg/L*hr)	Vz/F obs (L)	Cl/F obs (L/hr)
153	0.23	3.04	0.0	3.00	41.85	218.02	278.52	23.58	5.39
154	0.13	5.27	0.0	2.02	53.42	192.34	282.45	40.38	5.31
155	0.15	4.46	0.0	2.00	49.68	259.55	389.99	33.50	5.21
156	0.14	4.90	0.0	1.00	45.44	233.36	351.61	30.13	4.27
158	0.18	3.93	0.0	1.00	41.74	212.13	288.42	29.52	5.20
159	0.07	9.41	0.0	2.50	73.63	441.95	1050.02	25.87	1.90
160	0.26	2.68	0.0	3.00	68.87	354.90	441.29	12.15	3.40
161	0.14	4.81	0.0	0.50	56.32	270.22	400.43	25.97	3.75
162	0.15	4.66	0.0	1.52	52.68	273.54	418.01	24.14	3.59
163	0.09	8.16	0.0	1.50	57.56	326.32	710.58	24.83	2.11
164	0.16	4.35	0.0	1.52	58.38	335.51	451.98	27.74	4.43
165	0.09	7.70	0.0	2.50	49.01	275.27	559.16	29.80	2.68
166	0.39	7.45	0.0	2.02	64.6.	490.15	903.46	17.85	1.66
167	0.13	5.30	0.0	2.03	42.27	231.93	373.57	40.94	5.35
168	0.1.	6.09	0.0	2.00	51.44	246.59	427.71	41.1'	4.68
169	0.10	6.63	0.0	2.50	52.59	325.22	616.52	23.19	2.43

Data summary

	K (hr⁻¹)	half-life (hr)	tlag *min (hr)	tmax (hr)	Cmax (mg/L)	AUCt (mg/L*hr)	AUCi (mg/L*hr)	Vz/F obs (L)	Cl/F obs (L/hr)
N	141	141	142	142	142	141	141	141	141
median	0.12	5.89	0.0	1.98	52.70	288.41	499.68	25.73	3.78
5th centile	0.07	3.88	0.0	0.50	32.87	185.05	276.14	13.42	1.38
95th centile	0.18	9.74	0.0	3.03	77.08	441.04	967.05	41.24	5.39

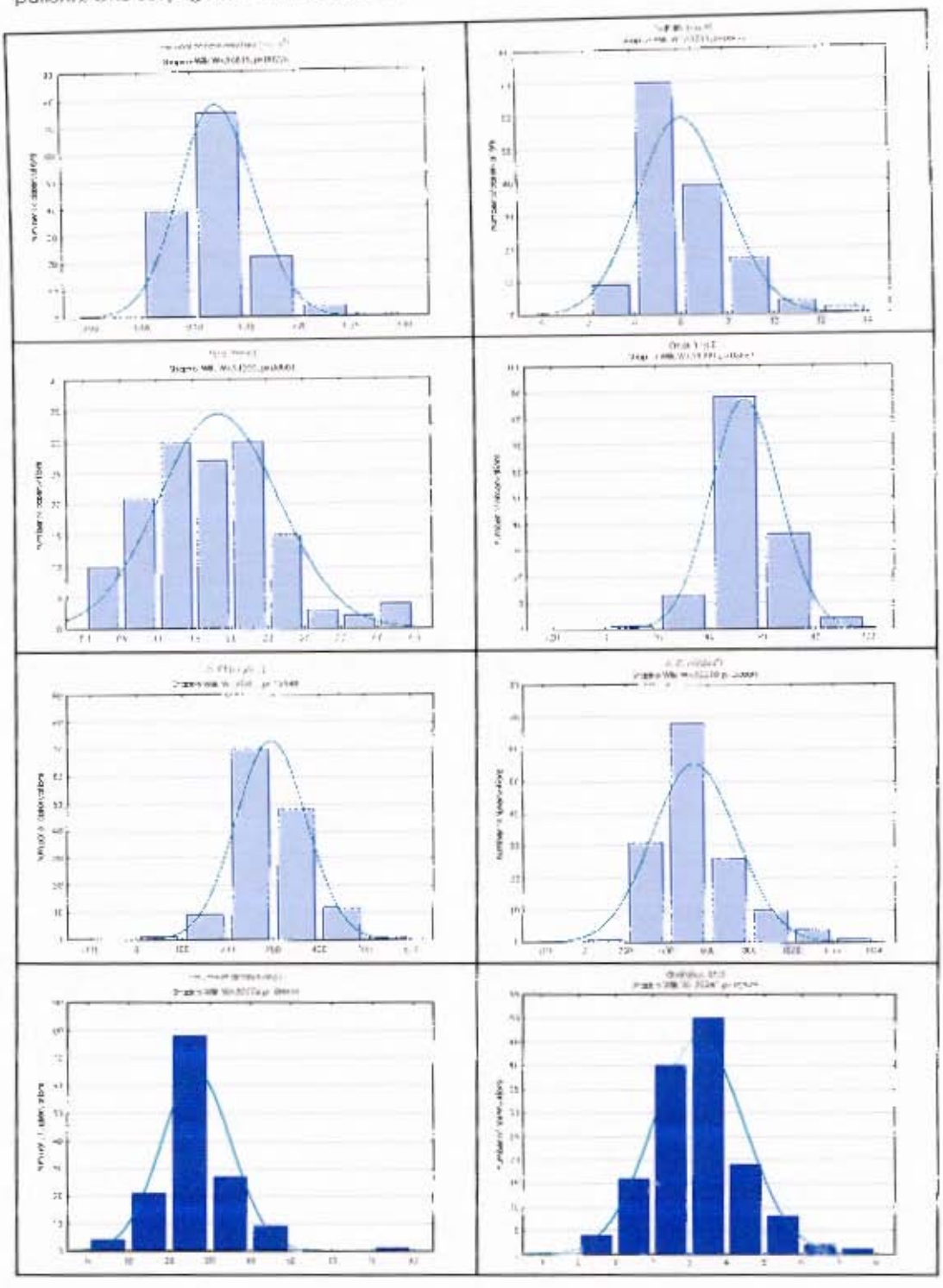
* Data excluded from further analysis as sampling was only available until 3 hours after drug administration.
** Outlying Vz/F obs and Cl/F obs values due to very low AUC; excluded from summary statistics.

Half-hourly sampling from 0 to 3 hours provided fairly accurate determination of the peak concentration of pyrazinamide as 126 (89%) of cases had peak concentrations lying between 0 and 3 hours.

The ratio of AUCt to AUCi was below 0.6 in 56% of the cases. In only one case was the ratio greater than 0.8. The ratio ranged from 0.33 to 0.81. This indicates that the duration of the sampling schedule of 6 hours was sub-optimal for characterizing pyrazinamide concentrations during the elimination phase, and that the values for the AUC, rely to a large extent on extrapolation.

The distribution of each pharmacokinetic measure is illustrated in figure 8. The Shapiro-Wilk test for normality showed that all the pharmacokinetic measures were skewed.

Figure 8: Histograms for pyrazinamide pharmacokinetic measures showing the distribution of the data in 142 patients. One outlying observation (subject 87) was omitted for volume of distribution and for clearance.



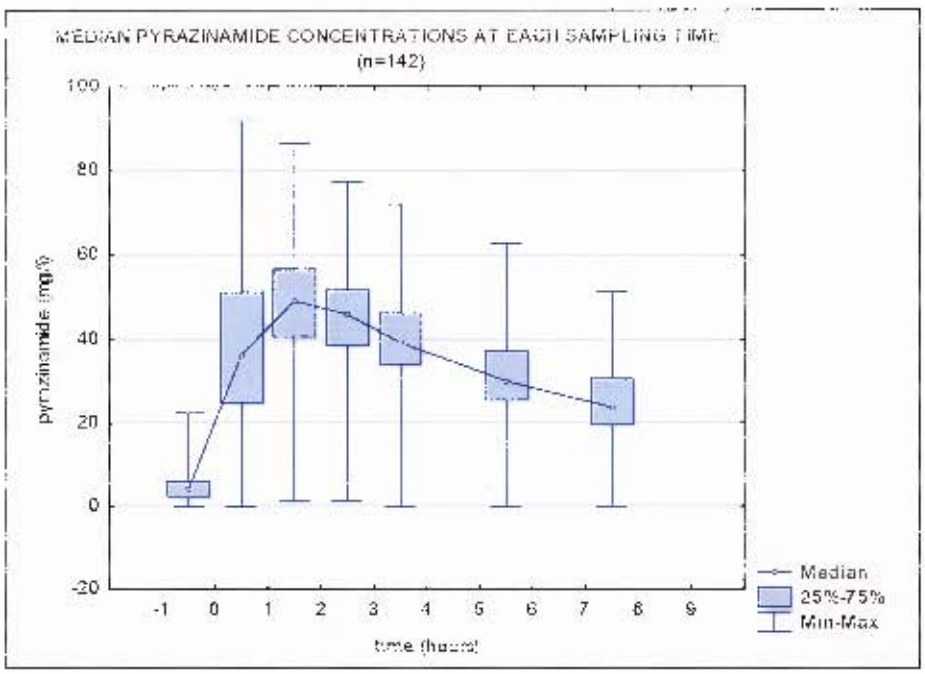
As demonstrated by the narrower interquartile ranges in figure 9, less variability was seen in the pyrazinamide concentrations between individuals than for rifampicin and isoniazid. The drug was absorbed reliably and rapidly; a lag time was not measured in

any patient and the median time to reach the peak concentration was 1.98 hours (95% CI: 1.5 – 2).

The median Cmax was 52.70 mg/l (95% CI: 51.12 – 55.20). Ninety-one patients (64%) had Cmax values above the published 30 – 50 mg/l range resulting from a 20 – 25 mg/kg dose, and only 4 patients (3%) had values below this range. The increased peak concentrations in this group of patients occurred in spite of more rapid elimination than trial suggested in the literature (half-life median of 5.89 hours, compared to 9 – 24 hours[128,137,185], and is probably largely due to relatively high doses per kilogram of body weight (mean 35.412 mg/kg; sd 5.86). The median AUC1 was 288.41 mg.hr.l⁻¹ (95% CI: 273.92 – 302.13). The AUCi was considerably greater (499.68 mg.hr.l⁻¹; CI: 454.67 – 522.88) as the drug was incompletely eliminated at 8 hours.

The median volume of distribution was 25.73 l (95% CI: 24.34 - 27.44). However, as the fractional absorption (F) is unknown, the V/ Fobs and Cl F obs values, respectively, do not represent true volume of distribution and clearance values.

Figure 9: Median concentration vs. time curve for pyrazinamide



The pyrazinamide concentrations at each sampling time are summarized in table 12.

Owing to the relatively long half-life of pyrazinamide, residual concentrations of the drug (from the previous dosing interval) were detected at the pre dose sample.

The 2 hour sampling time correlated best with the peak concentration, and was the mode for Tmax.

Table 12: Pyrazinamide concentrations at each sampling time and the correlation with the peak concentrations.

Time of sampling (hr)	N	Median concentration (mg/l)	Centiles (mg/l)				Time=Tmax n	Correlation Cmax vs. conc. or time[2]
			0	5[1]	95[1]	100[1]		
0.0	142	3.92	0	0.81	13.32	52.45	0	0.450(0.0000)
0.5	142	29.94	0	8.60	66.04	91.84	12	0.463(0.0000)
1.0	142	44.57	1.41	18.38	69.25	78.61	25	0.772(0.0000)
1.5	142	48.81	1.42	26.27	71.81	82.34	31	0.866(0.0000)
2.0	142	49.62	2.27	28.15	73.76	83.27	34	0.859(0.0000)
2.5	142	47.49	1.35	27.46	68.19	76.83	22	0.837(0.0000)
3.0	142	44.28	1.25	27.36	67.41	77.24	10	0.790(0.0000)
4.0	141	39.27	1.10	22.17	61.25	72.10	2	0.736(0.0000)
5.0	141	30.48	1.07	18.93	49.89	62.67	0	0.707(0.0000)
8.0	141	23.62	0	13.03	42.31	51.18	0	0.657(0.0000)

[1] samples test for 1 subject
[2] spearmans rho (p value)

Correlations between the various pharmacokinetic measures were explored. The results are illustrated in table 13.

Table 13: Sagarman's correlations between the various pharmacokinetic measures of pyrazinamide expressed by correlation coefficient (p value).

	Tlag							
Tmax	-	**Tmax**						
Cmax	-	0.2868 (0.0035)	**Cmax**					
K	-	-0.0638 (0.4524)	0.7458 (0.0845)	**K**				
T1/2		0.0636 (0.4539)	0.1460 (0.0841)	-1.0000 (0.0000)	**T1/2**			
AUCt	-	-0.1205 (0.1347)	0.8764 (0.0000)	0.3732 (0.0000)	0.3732 (0.0000)	**AUCt**		
AUCi		0.0269 (0.7548)	0.6537 (0.0000)	-0.7759 (0.0000)	0.7259 (0.0000)	0.8736 (0.0000)	**AUCi**	
VzF	-	-0.0304 (0.3422)	0.4664 (0.0000)	0.1395 (0.0990)	0.1395 (0.0989)	-0.3833 (0.0000)	-0.3077 (0.0002)	**VF**
ClF		-0.0502 (0.4780)	-0.4739 (0.0000)	0.5994 (0.0000)	0.5993 (0.0000)	0.6736 (0.0000)	-0.8026 (0.0000)	0.6307 (0.0000)

Greater Tmax values were significantly correlated with lower peak pyrazinamide concentrations. Delayed absorption was therefore associated with lower peak values as in the case of rifampicin. However a significant correlation was not seen between Tmax and the AUC values, indicating that delayed absorption was not a strong determinant of overall bioavailability over time.

Cmax and AUCt were strongly and highly significantly correlated, and the 2 measures were highly significantly associated with the AUCi, although the correlations with AUCi were weaker. More of the variability in AUCi is explained by the rate of elimination.

than is the case for AUCt; the elimination rate constant and the half-life were significantly associated with the AUC values, with the stronger relationship being displayed with AUCi.

While the Vz F obs and Cl F obs values showed a greater degree of independence from the markers of bioavailability, Cmax and AUC, than they did for rifampicin or isoniazid, they were still significantly associated with these measures and are not adjusted for the fractional absorption. They cannot therefore be regarded as true reflections of the volume of distribution and clearance, respectively.

ETHAMBUTOL

The pharmacokinetic measures of ethambutol derived from the individual concentration-time profiles are tabulated below (Table 14).

Table 14: The elimination rate constant (k), half life, lag time, time to reach the peak concentration (Tmax), the peak concentration (Cmax), the area under the concentration time curve to the last measurable concentration (AUCt), the area under the concentration-time curve to infinity (AUCi), the volume of distribution (Vz F obs) and the clearance (Cl F obs) of ethambutol for each subject, 2 months after admission to Stewelsdoot Hospital

Subject	K (hr⁻¹)	half-life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg.l⁻¹)	AUCt (mg.l⁻¹.hr)	AUCi (mg.l⁻¹.hr)	Vz F obs (l)	Cl F obs (l.hr⁻¹)
1	0.29	2.37	0.0	3.00	3.27	13.76	17.26	178.63	46.35
2	0.27	2.59	0.0	4.00	4.28	16.31	20.79	186.39	50.44
3[b]	-	-	-	-	-	-	-	-	-
4	0.26	2.67	0.0	3.00	1.27	6.40	7.85	399.28	101.91
5	0.34	2.05	0.0	4.00	4.25	16.99	20.30	175.97	59.12
6	0.31	2.27	0.0	3.18	4.17	15.64	18.76	203.88	63.95
7	0.50	1.39	0.0	2.50	2.16	6.65	6.93	347.91	173.68
9	0.65	1.07	0.0	2.50	5.53	20.07	20.54	90.22	58.41
10	0.37	1.89	0.0	3.02	6.38	22.99	25.86	125.81	46.25
11	0.30	2.34	0.0	2.00	5.31	19.02	22.50	183.77	54.46
13	0.32	2.19	0.0	4.03	5.85	18.09	23.58	160.95	50.90
14	0.28	2.48	0.0	2.50	3.50	12.76	15.85	271.32	75.71
15	0.17	4.19	0.0	3.00	4.83	21.49	31.62	229.78	38.96
16	0.24	2.94	0.0	2.00	4.17	15.18	17.96	279.80	66.87
17	0.25	2.87	0.0	3.00	5.38	17.51	21.32	157.48	37.52
18	0.26	2.65	0.0	1.50	4.49	11.60	13.51	220.04	57.50
19[a]	-	-	-	-	-	-	-	-	-
20[a]	-	-	-	-	-	-	-	-	-
22[a]	-	-	-	-	-	-	-	-	-
23	0.06[b]	11.[illegible]	0.0	3.00	3.65	21.61	57.95[c]	33.87	20.71
24	0.33	2.09	0.0	2.98	7.50	31.89	37.01	97.53	32.42
25	-	-	0.0	6.08	4.47	25.57	-	-	-
26	0.61	1.14	0.0	3.00	5.90	24.28	25.06	52.35	31.92
27	0.25	2.80	0.0	2.98	8.61	28.16	35.38	86.12	30.31
29	0.25	2.76	0.0	4.03	3.52	5.82	20.99	157.00	38.12
30	0.27	2.54	0.0	1.50	4.69	14.13	16.09	192.22	49.72
31	0.35	2.01	0.0	3.00	6.49	21.65	24.87	93.06	32.17
34	0.24	2.87	0.0	2.52	5.04	23.17	29.84	111.09	26.81
37	0.37	1.87	0.0	1.98	9.79	24.04	26.30	120.61	44.27
41	0.15	4.63	0.0	1.05	7.97	23.43	30.81	260.08	38.95
42	0.19	3.67	0.0	4.00	3.55	16.62	25.33	247.74	47.98
43	0.23	2.97	0.0	4.00	4.56	19.37	27.21	188.80	44.17
44	0.21	3.25	0.0	4.00	4.56	20.90	30.15	124.90	26.53
45	0.33	2.13	0.0	2.50	3.94	17.22	19.56	188.75	61.34
46	0.23	3.06	0.0	4.00	5.70	23.24	33.57	158.73	35.97

Table 4 continued

	K (h⁻¹)	half-life (h)	lag time (h)	tmax (h)	Cmax (mg l⁻¹)	AUC0-t (mg · h/l)	AUC0-∞ (mg · h/l)	Vz/F obs (l)	CL/F obs (l h⁻¹)
47	0.37	1.87	0.0	4.00	4.06	18.51	20.93	154.28	57.32
48	0.46	1.51	0.0	4.00	4.49	15.89	17.45	150.06	68.77
49	0.52	1.33	0.0	1.58	5.24	17.94	18.59	129.82	64.57
50	0.45	1.55	0.0	4.00	6.24	24.85	27.15	95.78	29.46
51	0.33	2.13	0.0	4.00	4.62	19.96	23.73	155.38	50.56
52	-	-	0.0	5.00	8.21	35.84	-	-	-
53	0.21	3.30	0.0	3.00	3.24	14.27	18.73	267.81	64.05
54	0.19	3.59	0.0	4.00	1.79	9.80	13.88	447.89	86.44
55	0.23	2.97	0.0	1.50	4.73	16.76	20.30	369.33	39.40
56	0.44	1.58	0.0	1.50	6.47	18.65	19.47	93.91	41.08
57	0.18	3.82	0.0	1.50	2.89	10.33	14.31	445.66	81.05
58	0.27	2.55	0.0	2.50	2.87	11.79	14.16	207.69	56.49
59	0.37	1.86	0.0	2.50	3.70	16.32	18.18	177.26	66.02
60	0.28	2.46	0.0	4.00	4.25	16.42	21.30	199.97	55.35
61	0.18	3.87	0.0	2.50	2.59	10.52	15.43	407.76	73.03
63	0.26	2.70	0.0	3.98	4.40	19.44	25.44	180.84	47.17
64	0.24	2.9	0.0	.02	7.xx	26.03	33.77	150.61	30.33
65	0.21	3.25	0.0	1.50	6.73	17.50	20.55	181.78	38.74
66	0.20	3.51	0.0	3.00	6.41	28.14	37.35	199.60	32.13
67	0.36	1.94	0.0	3.03	10.44	45.23	50.90	65.56	23.58
68	0.54	1.29	0.0	3.67	4.14	16.93	17.57	126.88	68.29
70	0.43	1.61	0.0	3.06	3.63	13.25	14.15	187.85	84.82
7	0.47	1.47	0.0	4.03	3.84	17.90	19.15	133.10	62.67
72	0.58	1.20	0.0	2.00	4.99	10.40	15.15	197.13	79.22
73	0.48	1.45	0.0	4.05	4.54	17.98	19.37	120.13	61.94
74	0.21	3.30	0.0	3.05	4.46	22.78	29.57	193.02	40.58
75	0.25	2.76	0.0	1.00	5.47	19.80	24.34	196.47	49.29
77	0.33	2.09	0.0	2.53	5.51	16.22	19.23	188.02	62.42
80	0.25	2.77	0.0	3.28	7.87	34.33	38.49	177.80	43.26
81	0.09	8.11	0.0	.98	4.65	18.43	32.79	428.08	36.50
83	0.24	2.85	0.0	2.00	4.00	19.84	25.05	197.15	47.90
84	-	-	-	-		-	-	-	-
85	0.24	2.84	0.0	1.02	2.15	10.21	12.89	253.93	62.05
87	-	-	0.0	3.23	0.19	0.20	-	-	-
88	0.23	3.00	0.0	3.02	8.37	29.58	36.80	141.30	32.61
89	0.54	1.28	0.0	4.00	4.47	17.03	17.97	123.59	66.77
90	0.27	2.56	0.0	4.00	4.50	18.42	23.89	185.05	50.24
91	0.44	1.58	0.0	4.00	7.66	28.32	31.24	87.30	38.42
92	0.28	2.49	0.0	3.00	3.05	14.54	17.77	247.63	67.52
93	0.19	3.65	0.0	3.00	6.66	31.58	43.57	96.76	18.36
94	0.44	1.58	0.0	3.02	4.96	15.86	14.86	107.94	47.45
95	0.20	3.38	0.0	3.03	6.43	26.03	33.36	175.66	35.57
96	0.24	2.85	0.0	2.50	3.69	13.92	16.84	292.65	71.28
97	0.38	1.82	0.0	3.00	6.50	22.70	25.36	124.15	47.39
101	0.29	2.41	0.0	2.00	6.11	26.17	31.29	133.37	38.36

Table 14 continued

	k (h⁻¹)	half life (hr)	lag time (hr)	Tmax (hr)	Cmax (mg/L)	AUC (mg·hr/L)	AUC (mg·hr/L)	Vd (L)	Clearance (L/hr)
103	0.40	1.72	0.0	2.50	6.96	18.04	20.44	143.85	58.70
104	0.19	3.57	0.0	1.52	5.76	21.44	26.67	231.75	44.99
105[a]			-	-	-	-	-	-	-
106	0.20	3.55	0.0	4.02	5.79	22.62	36.18	113.16	22.11
107[a]	-	-	-	-	-		-	-	-
108	0.24	2.92	0.0	2.50	4.50	15.41	24.83	135.95	32.22
109	0.29	2.35	0.0	4.00	4.06	21.17	25.4	160.74	47.23
110	0.17	4.07	0.0	2.00	4.55	17.68	23.18	222.58	34.52
111	0.27	2.58	0.0	2.52	4.52	20.69	24.92	149.57	40.13
112	0.13	5.15	0.0	2.00	4.72	20.18	40.42	275.69	37.11
113	0.24	2.93	0.0	3.00	4.79	26.08	33.88	149.88	35.42
114	0.24	2.89	0.0	2.52	4.41	20.33	25.99	192.57	46.18
115	0.14	4.80	0.0	2.53	5.44	28.84	45.66	282.02	26.28
116[a]		-	-	-			-	-	-
117[a]	-	-	-				-	-	-
118[a]	-	-	-	-	-	-	-		-
119	0.27	2.57	0.0	2.02	4.11	21.37	24.95	158.27	40.07
120	0.28	2.57	0.0	4.00	6.05	24.65	31.96	136.40	37.55
121	0.34	2.03	0.0	2.50	4.84	18.34	20.60	142.35	48.55
122	0.28	2.47	0.0	3.03	3.98	19.93	24.50	174.69	48.98
123	0.31	2.21	0.0	1.55	3.97	15.74	17.71	179.71	56.48
124	0.22	3.17	0.0	2.10	3.76	7.86	21.99	249.59	54.57
125	0.30	2.33	0.0	2.53	4.56	24.67	28.39	167.01	42.28
126	0.19	3.64	0.0	3.06	6.24	29.18	32.32	160.29	30.52
127[a]			-	-				-	-
128	0.28	2.46	0.0	3.03	6.68	24.64	30.64	115.70	32.63
129	0.22	3.12	0.0	3.00	5.35	24.95	32.37	138.18	29.72
130[a]	-		-	-	-	-	-	-	-
131	0.24	2.90	0.0	2.50	4.67	26.48	33.36	125.40	29.98
132	0.11	6.41	0.0	3.03	2.93	13.24	25.70	431.96	46.70
133	0.30	2.30	0.0	2.00	5.69	24.12	27.43	145.40	43.75
134	0.15	4.63	0.0	2.52	6.07	29.24	32.59	267.83	39.92
135	0.23	2.96	0.0	2.00	6.03	24.54	32.58	157.06	36.83
136	0.32	2.19	0.0	1.52	5.63	19.99	22.33	127.34	44.79
137[a]	-	-	-	-	-	-	-	-	-
138	0.19	3.64	0.0	1.03	3.90	13.87	18.89	333.91	63.52
139	0.23	3.08	0.0	3.00	7.49	29.90	46.30	95.76	21.60
140	0.32	2.15	0.0	1.11	5.27	15.47	17.13	180.66	58.36
141	0.22	3.11	0.0	2.00	4.20	21.91	28.92	186.03	41.50
142	0.24	2.90	0.0	3.02	4.06	17.37	22.43	224.06	53.52
143	0.17	4.05	0.0	4.05	3.69	18.46	29.39	198.81	34.02
144	0.30	2.34	0.0	2.66	8.12	31.12	37.00	109.57	32.44
145	0.29	2.41	0.0	4.06	5.26	25.50	31.52	132.20	38.07
146	0.23	3.05	0.0	1.50	5.62	21.27	26.45	199.26	45.38
147	0.17	4.14	0.0	2.02	3.79	15.40	21.12	338.97	56.81

Table 14 continued

	K (hr⁻¹)	half-life (hr)	lag time (hr)	tmax (hr)	Cmax (mg/L)	AUC1 (mg/L.hr)	AUC∞ (mg/L.hr)	Vz/F obs (L)	CL/F obs (L.hr⁻¹)
152	0.15	4.68	0.0	1.50	7.56	22.46	30.29	267.47	39.62
154	0.27	2.59	0.0	4.00	8.45	32.46	42.76	97.43	23.38
154	0.30	2.29	0.0	3.00	5.42	23.77	27.85	118.88	35.91
155	0.23	2.97	0.0	3.00	6.29	21.69	27.74	185.33	43.25
156	0.080[a]	8.92[a]	0.0	3.00	2.97	10.69	24.94[c]	515.74	40.09
158	0.33	2.10	0.0	2.55	5.40	20.65	23.62	127.35	42.34
159			0.0	4.02	3.21	16.22			
160	0.19	3.58	0.0	4.00	7.26	36.65	53.97	174.99	22.23
161	0.19	3.57	0.0	1.00	3.74	14.91	18.98	271.57	52.70
162	0.26	2.64	0.0	2.02	8.30	24.43	28.32	134.29	35.31
163	0.37	1.89	0.0	3.02	5.03	20.85	23.06	142.19	52.03
164	0.35	1.99	0.0	2.06	5.06	18.5	22.35	154.07	53.69
165	0.35	2.00	0.0	2.00	7.22	22.86	25.94	133.39	46.26
166	0.22	3.1[a]	0.0	3.02	6.65	26.41	26.05	124.45	27.74
167	0.51	1.37	0.0	3.03	6.09	19.94	21.02	112.58	57.08
168	0.21	3.37	0.0	3.00	8.08	24.67	32.66	178.51	36.74
169	0.30	1.71	0.0	3.00	8.23	34.01	38.04	86.91	31.55

Data summary

	λz (hr⁻¹)	half-life (hr)	lag time (hr)	tmax (hr)	Cmax (mg/L)	AUC1 (mg/L.hr)	AUC∞ (mg/L.hr)	Vz/F obs (L)	CL/F obs (L.hr⁻¹)
n	123	123	129	129	129	129	123	123	23
median	0.26	2.64	0.0	3	4.96	19.94	24.87	160.29	44.99
5th centile	0.15	1.34	0.0	1.04	2.48	10.37	14.15	87.39	23.42
95th centile	0.52	4.63	0.0	4.04	8.33	32.18	42.30	384.27	78.51

Missing pharmacokinetic measures (other than those outlined in a, b and c; below) could not be calculated due to insufficient measurable drug concentration points.

[a] Data excluded from further analysis because the goodness of fit of the elimination rate constant to the data is poor (subject number 23 r²=0.38; 156: r²=0.19); and the values of λz and half-life are, as a result, outliers.

[b] Subjects did not receive ethambutol on the day of PK measurement.

[c] Plasma samples for subjects 118, 119 and 120 were lost prior to ethambutol concentration determination.

Ethambutol reached peak concentrations later than did rifampicin, isoniazid and pyrazinamide, although no lag time was evident. Consistent with the median Tmax of 3 hours (95% CI 2.52 – 3.00), only 46% of the 129 Cmax values fell between the 0 and the 3 hour sampling times when half hourly sampling occurred. However, 95% of the profiles peaked at or before the 4 hour sample, allowing reasonable confidence in the Cmax values obtained for most patients.

After exclusion of 4 subjects in whom the slope of the terminal elimination curve could not be determined, and the 2 subjects with data fitting the elimination rate constant poorly, 56 of 123 (46%) had AUCt : AUCi ratios of less than 0.8, and 15 (12%) of these

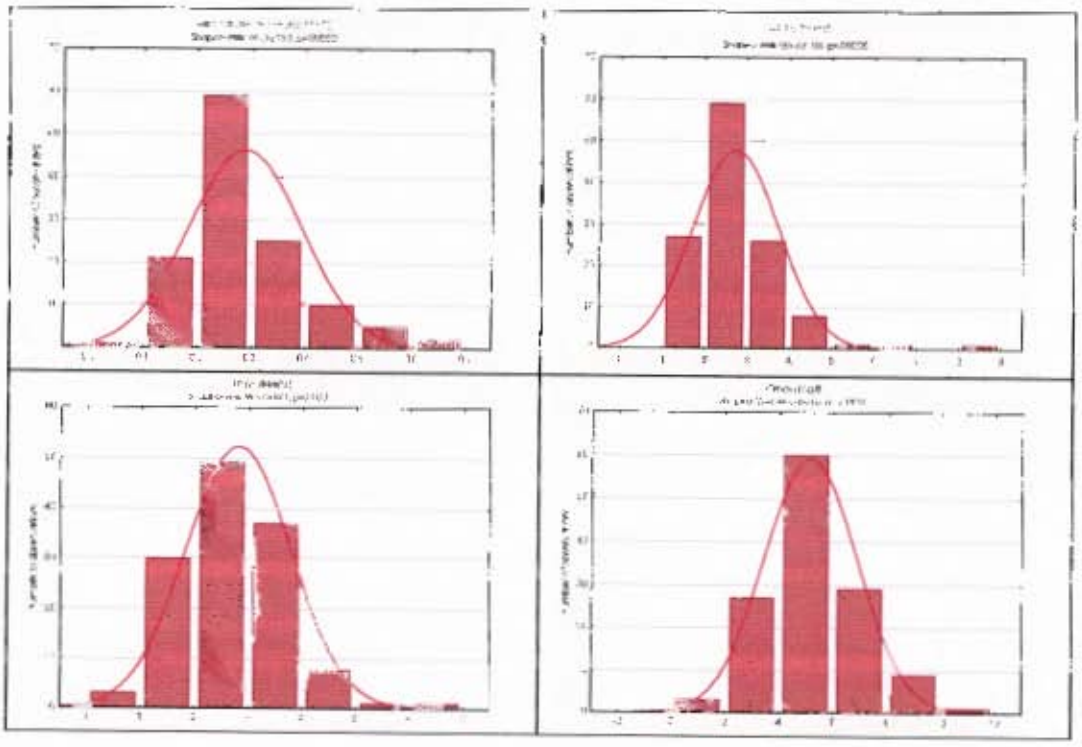
had ratios of less than 0.7. Therefore, in almost half of the subjects the AUCt value relies extensively on extrapolation.

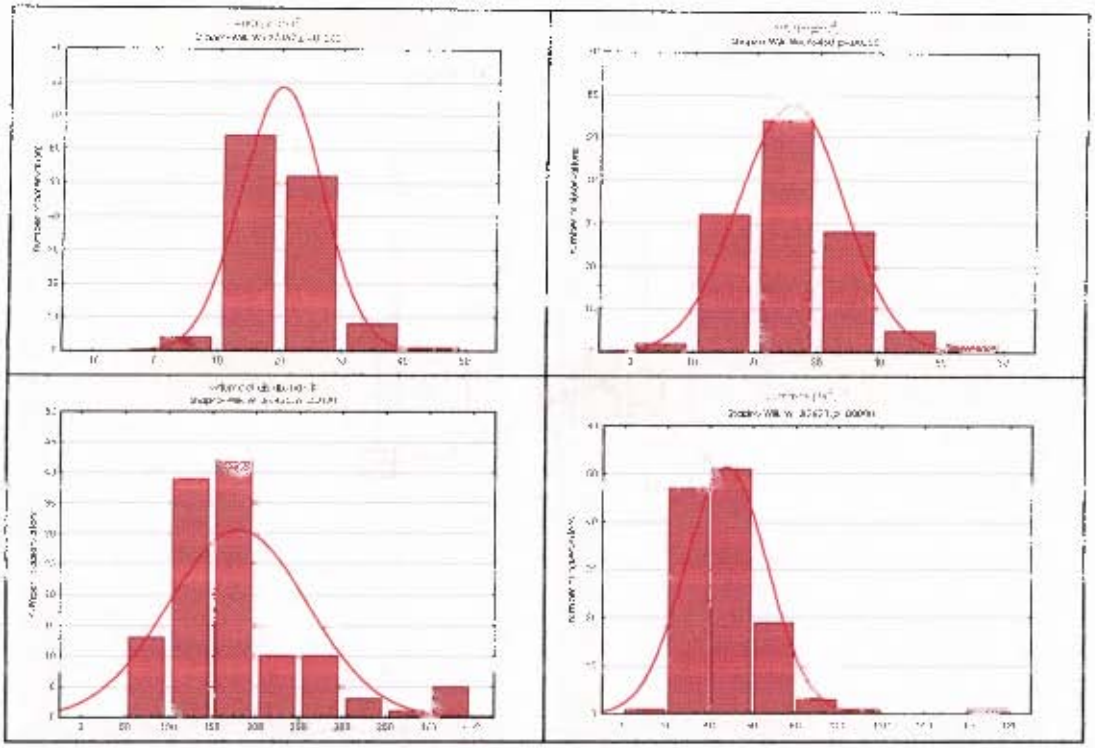
The maximum ethambutol concentration had a median value of 4.96 mg/l (95% CI 4.56 – 5.35). Three subjects (2%) had Cmax values lower than 2 mg/l, 22 (17%) had Cmax greater than 6 mg/l and the dose/kg ranged from 12.88 to 34.19 mg/kg. Based on the literature, a range of 2 – 6 mg/l after a single 25 mg/kg dose should be expected[56]. Thirty one subjects (24%) had 2-hour concentrations less than the proposed 2 mg/l level[56]. The median AUCt and AUCi were 19.94 mg.hr.l^{-1} (95% CI 18.43 – 20.92) and 24.87 mg.hr.l^{-1} (95% CI 22.51 – 25.94) respectively.

The half-life of ethambutol had a median of 2.64 hours (95% CI 2.46 – 2.85) and a range of 1.07 to 8.11 hours.

The median volume of distribution was 160.22 l (95% CI: 149.63 – 179.56), and the median clearance was 44.99 l.hr^{-1} (95% CI 40.64 – 47.44). However, as the fractional absorption (F) is unknown, the Vz Fobs and Cl F obs values, respectively, do not represent true volume of distribution and clearance values.

Figure 10: Histograms for each pharmacokinetic measure of ethambutol in 129 patients





As illustrated in figure 10, the Cmax of ethambutol had a normal distribution in the study population while the distribution of the other pharmacokinetic measures was skewed ($p<0.05$ using Shapiro-Wilks test for normally distributed data).

The median concentrations of ethambutol at each sampling are illustrated in figure 11.

The ethambutol concentrations at each sampling time are summarized in the table 15. The single time point that correlated best with the Cmax was at 3 hours.

Spearman's correlations between the various pharmacokinetic measures are shown in table 16. The weak positive correlation between Tmax and AUCt, and the negative correlation between volume of distribution and Tmax may be due to poor characterization of the terminal elimination curve in subjects with a late peak concentration, leading to spuriously low elimination constants. The strong correlation between Cmax and AUCt, and to a lesser extent AUCi is expected; they are all markers of bioavailability. The negative correlations of Cmax and AUC, with Vz F and Cl F are, in part, because the calculation of volume of distribution and clearance is not adjusted for variations in the bioavailability. These parameters are therefore unreliable markers of volume of distribution and clearance, per se, and are not used in further analyses.

Figure 11: The median concentration vs. time curve for ethambutol. Error bars indicate the ranges of concentrations at each sampling time and the boxes represent the 25% - 75% percentile ranges.

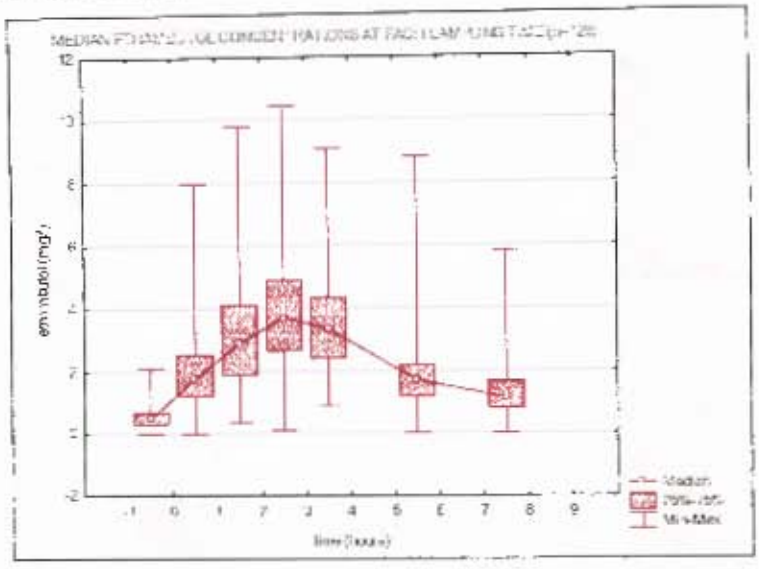


Table 15: Ethambutol concentrations at each sampling time and the correlation with the peak concentrations.

Time of sampling (hr)	N	Median concentration (mg/l)	Centiles (mg/l)				time - tmax	Correlation Cmax vs. conc. at time
			0	5%	95%	100%		
0.0	129	0.45	0	0	1.52	2.09	-2	0.3321(0.0001)
0.5	129	1.39	0	0.46	2.99	5.11	0	0.4586(0.0000)
1.0	129	2.16	0	0.87	5.50	7.93	8	0.4685(0.0000)
1.5	129	2.58	0	0.95	6.36	8.51	12	0.5198(0.0000)
2.0	[illegible]	3.07	0	0.78	6.55	9.79	16	0.5302(0.0000)
2.5	129	3.72	0.11	1.42	6.78	8.51	21	0.6576(0.0000)
3.0	129	3.70	0	1.27	7.69	10.44	39	0.6585(0.0000)
4.0	[illegible]	3.79	0	[illegible]	5.65	7.58	20	0.4541(0.0000)
6.0	129	1.65	0	0.65	3.80	8.51	2	0.4064(0.0000)
8.0	129	1.12	0	0.35	2.70	5.94	0	0.3509(0.0000)

(spearmans rho (p-value)

Table 16: Spearman's correlations between the various pharmacokinetic measures of ethambutol expressed by correlation coefficient (p-value).

	Tlag							
Tmax		Tmax						
Cmax		-0.1071 (0.2272)	Cmax					
K	-	0.1283 (0.1573)	0.5510 (0.5755)	K				
T1/2	-	-0.1285 (0.1564)	-0.0511 (0.5747)	1.0000 (0.0000)	T1/2			
AUCt	-	0.1339 (0.1329)	0.8005 (0.0000)	0.0916 (0.3146)	0.0913 (0.3152)	AUCt		
AUCi		0.1701 (0.0650)	0.6890 (0.0000)	-0.3793 (0.0000)	0.3792 (0.0000)	0.9214 (0.0000)	AUCi	
Vz/F	-	-0.2482 (0.0056)	0.1108 (0.0950)	-0.3211 (0.0000)	0.3210 (0.0000)	-0.3269 (0.0000)	-0.3985 (0.0000)	VF
Cl/F	-	-0.0990 (0.2780)	-0.6475 (0.0000)	0.3616 (0.0000)	-0.3615 (0.0000)	-0.8554 (0.0000)	0.8753 (0.0000)	0.5 34 (0.0000)

PATIENT PATTERNS

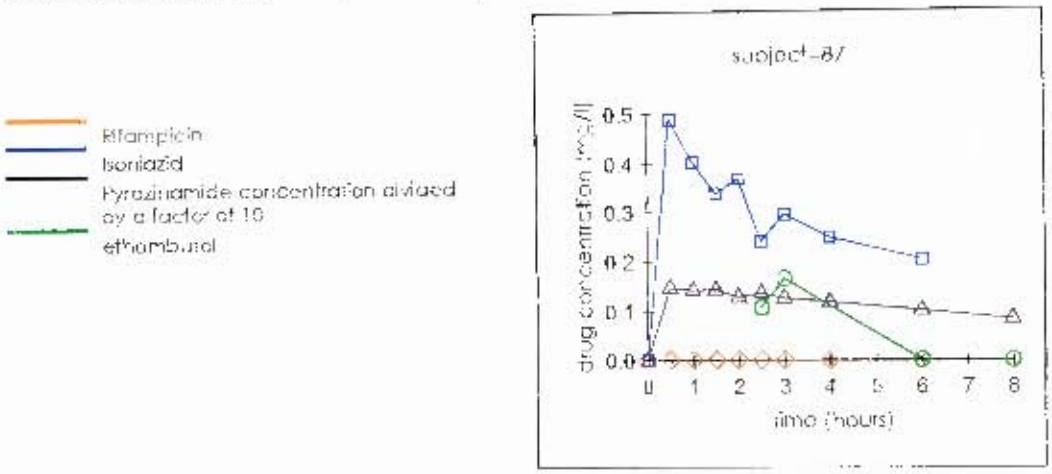
Certain subjects tended to achieve relatively low drug concentrations of more than one drug. Fifteen subjects had Cmax or AUC values of 3 or 4 drugs in the lowest 20% of the values recorded within the patient cohort. The drug concentration vs. time profiles for these subjects (16, 42, 43, 45, 49, 53, 61, 67, 87, 92, 110, 125, 135, 153, and 160) can be viewed in Appendix 5.2.

Eleven patients had peak concentrations of rifampicin less than 1 mg/l. All patients with rifampicin Cmax below 1 mg/l, received rifampicin containing products of batches not approved by the regulatory authority on the day of pharmacokinetic sampling. The majority of these patients achieved adequate concentrations of the other drugs measured. Patient 54 had a peak concentration of ethambutol of 1.70 mg/l, slightly below the range expected[56,57,66]. Subject 87 had particularly low levels of all 4 drugs, and the case deserves further discussion.

Subject 87 was 17 years old when she was first diagnosed with smear positive pulmonary tuberculosis in May 1999. Her previous medical history was unremarkable apart from treatment for symptomatic anaemia approximately 2 years previously. She was started on regimen 1 comprising rifampicin, isoniazid, pyrazinamide and ethambutol under the auspices of her local clinic. At the end of the 2 month intensive phase her sputum smear was negative, so pyrazinamide and ethambutol were dropped from the regimen and rifampicin and isoniazid were continued. However, by five months in spite of apparently excellent compliance she had gained only 2 kg of body weight, the smear was again positive and the culture grew *Mycobacterium tuberculosis* sensitive to rifampicin and isoniazid. She was transferred to Brewelskloof Hospital for unexplained treatment failure. Streptomycin was added to her regimen. After 2 months in hospital when the first pharmacokinetic assessment was made as part of this study, she had gained 6 kilograms and her sputum was smear and culture negative. The drugs were given on an empty stomach on the day of PK sampling. Rifampicin 450 mg (unfortunately in one of batches not approved by the regulatory authority), isoniazid 300 mg, pyrazinamide 1500 mg and ethambutol 1200 mg were administered as separate single drug formulations with a glass of water. Concomitant medications were daily streptomycin 750 mg intramuscularly, pyridoxine 25 mg daily and a tablet of iron sulphate 3 times daily.

The concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol vs. time for subject 87 are shown in the graph below (figure 12):

Figure 12: Drug concentration-time profiles for subject 87



At this stage the only remarkable clinical feature was microcytic anaemia (haemoglobin 8.0 g/l; MCV 62 fl), with a low serum iron (3.60 µmol/ l; normal range 8.10 – 30.40), and transferrin saturation (4.5 %; normal range 25 – 45 %), and normal serum transferrin (3.05 g/l; normal range 1.75 – 4.00) and serum ferritin (8 µg/l; normal range 7 – 282). The anaemia showed little improvement on antituberculosis therapy and iron supplementation. No abnormal blood loss was identified, although gastroscopy with antral and fundal biopsies found *Helicobacter pylori* gastritis which was treated with antibiotics and sucralfate.

The subject was discharged from Brewelskloof Hospital for a further 6 months of clinic-based treatment with rifampicin 600 mg, isoniazid 300 mg, pyrazinamide 1500 mg and ethambutol 800 mg, but no streptomycin. On completion of this treatment regimen she was again sputum culture positive, in spite of good compliance. The organisms cultured were, again, sensitive to isoniazid and rifampicin. She was continued on 4 drug treatment in hospital and drug concentrations were measured again on 2 occasions.

Further clinical details are not available, but 15 months after the previous admission she was found to be sputum culture positive for sensitive organisms once again, and drug levels were measured 6 months later on increased drug doses when she was still not responding satisfactorily to treatment.

Drug concentration determination was repeated by the study team at the request of the patient's doctor on 4 occasions after the initial measurements. On each occasion the drugs were given together under strict observation, on an empty stomach and with a glass of water.

The date of PK sampling and the drug details are shown in table 17.

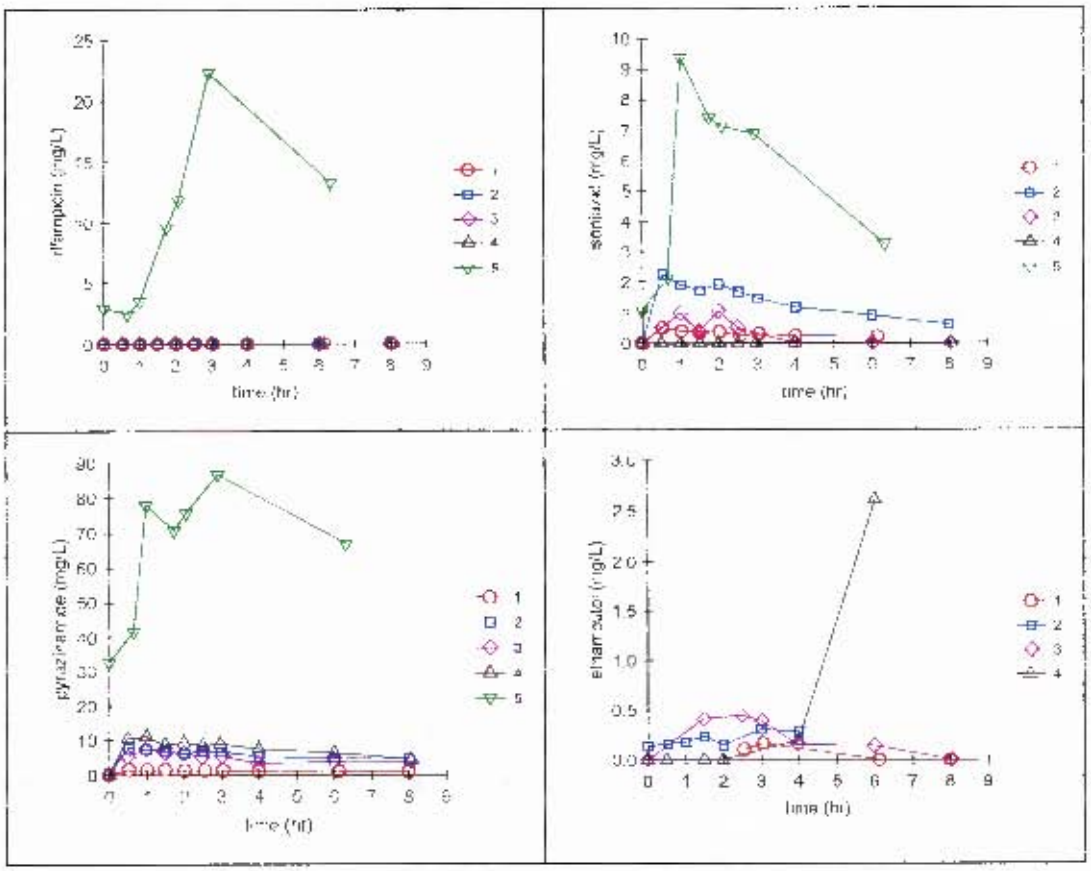
Table 17: Dates of PK sampling and antituberculosis drug details for subject 87.

Occasion	1 (study	2	3	4	5
Date	10/02/2000	30/03/2000	22/06/2000	07/12/2000	04/09/2002
Approximate time after previous dose	24 hours	24 hours	24 hours	24 hours	16 hours
Rifampicin	450 mg (NA)	600 mg	600 mg	600 mg (FDC)	900 mg
Isoniazid	300 mg	300 mg	300 mg	300 mg (FDC)	450 mg
Pyrazinamide	1500 mg	1500 mg	1500 mg	1500 mg	2000 mg
ethambutol	1200 mg	1200 mg	1200 mg	2000 mg	1200 mg
Streptomycin	750 mg	750 mg			750 mg

NA: product batches not approved by the regulatory authority; unmarked products were fully approved
FDC: Fixed dose combination; unmarked formulations were single drug formulations

The drug concentrations measured on each occasion are illustrated in figure 13.

Figure 13: Drug concentration-time profiles for rifampicin, isoniazid, pyrazinamide and ethambutol in subject 87 on 5 occasions.



Rifampicin concentrations remained undetectable at 450 mg and 600 mg doses. Repeated measurements confirmed that the low concentrations were not due to day-to-day variation. Measurement of rifampicin in a single 600 mg tablet on occasions 2 and 3, and as part of a fixed dose combination tablet on occasion 4 confirmed that 600 mg doses of a variety of approved products failed to achieve

detectable concentrations. In contrast, rifampicin concentrations after the 900 mg dose were high. It should be noted that on this occasion the drugs were given only 16 hours after the previous doses of the same amounts, and that this occasion was 21 months after occasion 4. Interestingly, Mehta et al. also found rifampicin concentrations to be increased in a non-linear manner amongst 6 subjects, in whom higher doses were given, after low concentrations had been detected on standard 600 mg doses[78] (table 18). The data suggests that a process, or processes, acting against absorption (possibly the efflux of P-glycoprotein) and, or, processes of metabolism and elimination may be saturated at higher doses.

Table 18: Rifampicin concentrations between 1.5 and 2.5 hours after drug administration in 6 subjects, at increasing rifampicin doses. (From Mehta JB, Shantaveerappa H, Byrd RP, Wu Fen SE, Tsenluan F, Roy TM. Utility of rifampicin blood levels in the treatment and follow-up of active pulmonary tuberculosis in patients who were slow to respond to routine directly observed therapy. Chest 2001; 120: 1520-1524)

Patient no.	Rifampicin concentration after 600 mg dose (mg/l)	Rifampicin concentration after 900 mg dose (mg/l)	Rifampicin concentration after 1500 mg dose (mg/l)
1	1.5	9.2	-
2	5.9	14.4	
3	< 1.0	9.9	
4	< 1.0	1.04	20.28
5	< 1.0	13.8	-
6	3.54	15.21	

Isoniazid concentrations on the first 4 occasions, after the 300 mg doses, were low. They were undetectable when the drug was given in the FDC in combination with rifampicin. The 400 mg dose on the last occasion produced good peak concentrations.

Similarly, pyrazinamide concentrations remained low after the 1500 mg doses but increased dramatically on the last occasion when the dose was increased to 2000 mg.

Ethambutol levels were not measured on the last occasion. However, very low levels resulted from the 1200 mg doses on the first 3 occasions. The 2000 mg dose on the 4th occasion produced an adequate but late peak concentration at 6 hours and the concentration had dropped below the validated range for the assay by 8 hours after dosing.

In summary, the case remains a mystery. It is likely that the improved response to treatment during her hospital admissions was related to intramuscular streptomycin administration. In the following chapter it can be seen that a low haemoglobin concentration was a risk factor for low concentrations of rifampicin, isoniazid and ethambutol, and microcytosis was a risk factor for delayed absorption of rifampicin

and isoniazid. However, this patient had few of the other risk factors identified for reduced drug bioavailability. It is possible that gastritis and, or, micronutrient deficiencies contributed to poor drug absorption but if these were the important determinants of drug absorption one would expect more patients in this cohort to have had very low concentrations of several drugs. However, the case suggests that there are certain patients in whom standard drug doses fail to reach adequate concentrations that the inadequate systemic drug exposure can result in treatment failure and disease relapse, and that high doses of orally administered drugs may overcome the markedly reduced bioavailability of the standard doses.

The individual patient drug concentration profiles over time are displayed in Appendix 5.2. In many patients the profiles of the different drugs can exhibit a similar shape, whilst in others no obvious pattern is evident. A substantial minority of the concentration - time profiles for rifampicin, isoniazid and ethambutol exhibited 2 peaks, which could exhibit entero-hepatic recirculation in the case of rifampicin or erratic drug absorption into the systemic circulation.

DISCUSSION

The pharmacokinetic characteristics of rifampicin, isoniazid, pyrazinamide and ethambutol were described in a large cohort of tuberculosis patients, using intensive sampling and noncompartmental analysis.

If the pharmacokinetics of the drugs have previously been measured in a patient cohort of this size, the findings are not in the public domain. Published data on ethambutol levels in patients is especially sparse. The study of a large cohort established the existence of a small but potentially important minority of individuals with very low or very high drug concentrations.

As for several other published patient studies (Table 2, Introduction), the drug concentrations measured in this study were clearly different to the reference ranges published in the literature. Rifampicin concentrations were generally lower, and those of isoniazid, pyrazinamide and ethambutol tended to be higher than the published ranges (table 19).

Table 19: A summary of the normal ranges in for the peak concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol in the study cohort.

	5th – 95th centile ranges for Cmax	Published ranges for Cmax[48]
Rifampicin (approved formulations only)	2.35 – 13.24 mg/l	8 – 24 mg/l
Isoniazid	3.55 – 12.07 mg/l	3 – 6 mg/l
Pyrazinamide	32.87 – 77.08 mg/l	20 – 60 mg/l
Ethambutol	2.48 – 6.33 mg/l	2 – 5 mg/l

The low concentrations of rifampicin achieved in many patients are cause for concern. Rifampicin concentrations were also inherently unpredictable. As only a single dosing occasion was observed, it is not known whether intra-individual variability (or within subject, day-to-day variability) contributed substantially to the variation observed between subjects. Conversely, most patients achieved adequate or high concentrations of isoniazid, pyrazinamide and ethambutol compared to published reference ranges.

Estimation of systemic rifampicin levels by quantification of rifampicin in urine collections may provide a useful screening tool for identifying patients with low levels. Similarly, visual inspection of urine with comparison to a colour chart was used for detecting treatment adherence in Malawi[45]. However, a single plasma concentration after 1 hour reflected Cmax and AUC values more accurately. Due to

time constraints, the amounts of urinary isoniazid, pyrazinamide and ethambutol were not determined.

Clearly those single drug rifampicin-containing product batches that were not approved by the regulatory authority had inferior bioavailability. Importantly, these batches had dissolution profiles in line with the stipulated requirements and had undergone, putatively, minor manufacturing changes. (Appendix 8). Nonetheless, very low rifampicin concentrations were also demonstrated in a substantial proportion of patients who received fully approved batches, and there was wide variability of the rifampicin levels amongst patients receiving fully approved batches.

While systemic drug exposure over a single dosing interval was accurately described for rifampicin and isoniazid, the concentration-time curves for pyrazinamide and ethambutol relied more heavily on extrapolation due to a relatively long half-life and late Tmax, respectively. Noncompartmental analysis relies largely on the measurement of actual drug concentrations rather than prediction of concentrations from a model. Thus, some measures contain variability attributable to the experimental design rather than reality. For example, Cmax is reflected more accurately in patients, who, by chance, achieved maximal systemic drug concentrations at a sampling time, than in patients who achieved maximal drug concentrations between 2 sampling times. The relatively intensive sampling schedule reduced this source of error. Variation due to experimental error was minimized by the use of standardized procedures, but can never be entirely excluded. More important limitations in describing drug exposure relate to the quantification of drug concentrations on a single occasion only (i.e. intra-individual variation was not quantified). Also, concentration measurements were limited to plasma in the systemic circulation (and urine, for rifampicin), while the concentration at the site of infection is more directly relevant to drug efficacy.

Patient and drug product factors associated with variation in the drug concentrations are explored in the next chapter. The impact of drug concentration (represented by the Cmax, AUC1 and AUCi values for each drug) on treatment response is reported in Chapter V.

IV

PATIENT AND TREATMENT FACTORS ASSOCIATED WITH PHARMACOKINETIC VARIABILITY

If clinically important variability occurs between the drug concentrations in different individuals then it may be useful to determine the association of the variability with easily identified covariate factors. In this chapter summary statistics of patient and drug covariate factors in the study population are presented. Before linear or logistic multiple regression models describing the covariate factors accounting for the variability associated with the pharmacokinetic measures of each drug.

COVARIATES

The 142 patients with drug-sensitive tuberculosis who were entered into the study were characterized according to the factors listed in the summary table below (table 1). Patient demographic features, drinking and smoking habits and treatment histories were obtained by means of a standard questionnaire completed by the ward doctor. At the same interview, approximately 2 months after admission to the hospital, patients were weighed and their height measured. Blood tests to determine the chemical pathology and haematology profiles, as well as HIV-infection status, were drawn on the day of blood sampling for the pharmacokinetic (PK) profiles of rifampicin, isoniazid, pyrazinamide and ethambutol (2 months after admission to Brewelskloof Hospital). At the same time a blood sample was drawn to determine acetylator type amongst a subgroup of patients. The drug doses and drug formulation details were obtained on the day of blood sampling for the PK profiles, by examination of the patients' drug dispensing charts, the drug dispensing carts in the wards and direct observation of the doses administered. The drug details recorded apply to the day of PK profile sampling.

Even though they had been treated for tuberculosis in hospital for 2 months prior to the study observations, many patients had clinical and laboratory features of systemic illness and chronic infection, including low body mass indices, low albumin, haemoglobin and creatinine levels; and elevated total protein levels, platelet counts and erythrocyte sedimentation rates (ESR). Chronic drug exposure may be partly responsible for the elevated ALT, AST, AP and γ-GT levels observed in the study cohort.

Table 1: Covariate factors characterizing the study patient's and the drugs they took on the day of PK sampling.

Patient factors:				
Demographic and clinical characteristics:	*n*	*Proportion(s)*		
sex	142	78 females (55%)	64 males (45%)	
alcohol*	139	82 (59%)		* regular consumption in the year before admission
smoking*	141	98 (70%)		
new / retreatment	142	51 new (36%)	91 retreatment (64%)	New: patients who had received less than one month of TB treatment prior to admission; retreatment: patients who had been treated for TB previously, or received ≥ 1 month of treatment prior to admission
Acetylator type	93	17 slow (18%)	76 intermediate or rapid (82%)	In 6 subjects genotyping was unsuccessful – phenotype was therefore used. In 4 subjects the phenotypes and genotypes were discordant: in these cases the genotype was used.
HIV-infection	141	14 (10%)		
	n	*Median*	*5th–95th centile:*	
age (years)	141	36	21 - 59	
weight (kg)	139	46	34 - 62	
body mass index (kg.m^{-2})	122	17.9	13.7 – 22.8	
Chemistry:	*n*	*Median*	*5th–95th centile:*	*normal ranges*
urea (mmol/l)	142	3.3	2.2 – 5.7	1.7 – 6.7
creatinine (µmol/l)	140	74	53 – 98	75 – 115
total protein (g/l)	142	78	68 – 94	60 – 80
albumin (g/l)	142	34	24 – 43	35 – 50
ALT (units/l)	142	14	8 – 40	1 – 25
AST (units/l)	142	17	11 – 40	7 – 25
AP (units/l)	141	68	43 – 156	30 – 70
γ-GT (units/l)	142	29	12 – 104	0 – 40
total bilirubin(µmol/l)	142	6	2 – 13	1 – 17
Haematology:	*n*	*Median*	*5th–95th centile:*	*normal ranges*
haemoglobin (g/dl)	142	13.9	8.4 – 15.3	♂: 13.3 - 17.3 ♀: 11.6 – 15.6
MCV (fl)	142	93	78 – 109	80 – 95
WCC (cells x 10^9/l)	142	8.5	4.2 – 15.7	4.0 – 11.0
Platelet count (x 10^9/l)	142	408	206 – 715	150 – 450
ESR (mm)	137	51	7 – 130	1 – 4
CD4+ cells/mm^3	124	699	196 - 1394	600 – 1200
Drug factors:				
Dose/kg: (weight not known for 3 subjects)				
	n	*Median*	*5th – 95th centile*	*recommended daily dose[1]*
Rifampicin (mg/kg)	139	10.90	8.82 – 14.20	10 mg/kg (maximum 600 mg daily)
isoniazid (mg/kg)	139	6.52	4.83 – 8.82	5 mg/kg (maximum 300 mg daily)
pyrazinamide (mg/kg)	139	35.71	25.21 – 47.32	20 - 35 mg/kg (maximum 3 g daily)
ethambutol (mg/kg)	131	24.49	16.84 - 32.58	15 -25 mg/kg
Formulation:	*n*	*Proportions*		
		Single drug formulations		*Fixed-dose combination products*
rifampicin	138[2]	Approved batches[3]: 55 (40%) Non-approved batches[3]: 54 (39%) Total: 109 (79%)		29 (21%)
isoniazid	138[2]	109 (79%)		29 (21%)
pyrazinamide	142	142 (100%)		
ethambutol	133[4]	133 (100%)		

Notes: [1] South African Medicines Formulary, 6th edition. Division of Pharmacology, Faculty of Health Sciences, University of Cape Town 2003.
[2] formulation not known, for 4 subjects
[3] 54 subjects received a single drug rifampicin formulation that was not approved by the regulatory authority on the day of PK profile determination[3]
[4] 9 subjects received drug regimens without ethambutol

Most of the covariate factor values had a skewed distribution. The distributions of albumin, mean cell volume, platelet count, and the doses per kilogram of isoniazid, pyrazinamide and ethambutol were more normally distributed (Shapiro-Wilk W test yields a p-value>0.05). The Chi², Kruskal-Wallis and Spearman's tests were used to explore the relationships between the covariate factors; the trends are illustrated in the tables 2a and 2b.

Table 2a: Association matrix of patient and treatment factors, displaying the statistical significance of the relationships.

p < 0.050	Kruskal-Wallis test p-value
0.050 ≤ p < 0.100	Pearson Chi2 test p-value
r: rifampicin H: isoniazid	Spearman's rank correlations were used if not otherwise specified: rho, followed by p-value

Due the large number of comparisons within the same patient sample it is likely that some of the weaker correlations are chance findings in this particular sample

Covariate factors:	Age	Sex	smoking	alcohol	Treatment category	formulation type	Formulation Regular/irregular	BMI	Acetylator type	HIV-infection	CD4+ cell count	Rifampicin dose/kg	Isoniazid dose/kg	pyrazinamide dose/kg	ethambutol dose/kg
Age															
Sex	K-W: .910														
Smoking	K-W: .309	Chi2: .911													
Alcohol	K-W: .534	Chi2: .342	Chi2: .000												
Treatment category	K-W: .861	Chi2: .000	Chi2: .671	Chi2: 0.5											
Formulation type (F, H)	K-W: .562	Chi2: .245	Chi2: .343	Chi2: .207	Chi2: .852										
R Formulation regular/irregular	K-W: .424	Chi2: .000	Chi2: .322	Chi2: .754	Chi2: .862	Chi2: .000									
BMI	-.021 .777	K-W: .055	K-W: .567	K-W: .317	K-W: .038	K-W: .594	K-W: .085								
Acetylator type	K-W: .986	Chi2: .575	Chi2: .155	Chi2: .210	Chi2: .155	Chi2: .511	Chi2: .060	K-W: .909							
HIV-infection	K-W: .208	Chi2: .696	Chi2: .022	Chi2: .308	Chi2: .051	Chi2: .045	Chi2: .343	K-W: .199	Chi2: .939						
CD4+ cell count	-.076 .865	K-W: .527	K-W: .002	K-W: .010	K-W: .480	K-W: .002	K-W: .085	-.139 .685	K-W: .093	Chi2: .003					
Rifampicin dose/kg	.037 .507	K-W: .534	K-W: .254	K-W: .735	K-W: .045	K-W: .938	K-W: .556	-.088 .000	K-W: .358	K-W: .068	Tau: .115				
isoniazid dose/kg	.035 .381	K-W: .804	K-W: .987	K-W: .735	K-W: .000	K-W: .915	K-W: .209	.765 .000	K-W: .021	K-W: .286	.178 .180	.564 .000			
Pyrazinamide dose/kg	.054 .256	K-W: .212	K-W: .964	K-W: .350	K-W: .094	K-W: .976	K-W: .000	.528 .000	K-W: .697	K-W: .191	.112 .216	.851 .001	.564 .000		
ethambutol dose/kg	-.141 .097	K-W: .112	K-W: .592	K-W: .629	K-W: .137	K-W: .646	K-W: .021	-.643 .000	K-W: .267	K-W: .315	-.057 .834	.575 .000	.56 .000	.154 .003	
Total protein	.147 .020	K-W: .000	K-W: .024	K-W: .267	K-W: .021	K-W: .000	K-W: .265	-.180 .042	K-W: .000	K-W: .001	-.171 .061	.581 .000	.089 .000	.047 .021	.047 .571
Albumin	-.124 .051	K-W: .932	K-W: .543	K-W: .730	K-W: .420	K-W: .055	K-W: .358	.599 .000	K-W: .365	K-W: .159	.099 .273	-.353 .000	.388 .000	-.346 .000	-.166 .051
Total bilirubin	.259 .401	K-W: .017	K-W: .463	K-W: .217	K-W: .267	K-W: .941	K-W: .008	.160 .026	K-W: .834	K-W: .934	.075 .975	.005 .969	.141 .099	.011 .909	-.022 .811
ALP	-.146 .195	K-W: .599	K-W: .972	K-W: .304	K-W: .033	K-W: .102	K-W: .029	.082 .316	K-W: .498	K-W: .090	-.008 .931	-.051 .549	.064 .450	.084 .327	.031 .716
ALT	-.108 .078	K-W: .599	K-W: .689	K-W: .457	K-W: .092	K-W: .435	K-W: .026	.240 .005	K-W: .767	K-W: .007	.050 .667	-.182 .051	-.121 .094	.211 .013	.064 .446
AP	-.127 .33	K-W: .274	K-W: .350	K-W: .039	K-W: .981	K-W: .299	K-W: .174	-.253 .002	K-W: .235	K-W: .674	.070 .465	.248 .009	.328 .000	.216 .017	.346 .000

Table 2a continued

Covariate factors:	Age	Sex	Smoking	Ethanol	Treatment category	Formulation type	Regulatory status	BMI	Acetylator type	HIV-infection	CD4+ cell count	Rifampicin dosing	Isoniazid dosing	Pyrazinamide dosing	Ethambutol dosing
γ-GT	.024 .383	K-W: .017	K-W: .469	K-W: .436	K-W: .242	K-W: .418	K-W: .017	.180 .042	K-W: .707	K-W: .060	K-W: .402	.173 .041	113 .165	.158 .063	.190 .034
[illegible]	.165 .049	K-W: .001	K-W: .503	K-W: .918	K-W: .757	K-W: .012	K-W: .746	.236 .003	K-W: .64	K-W: .516	K-W: .374	-.057 .061	-.157 .058	-.165 .018	-.146 .001
Creatinine	.072 .395	K-W: .955	K-W: .956	K-W: .106	K-W: .129	K-W: .085	K-W: .206	-.129 .156	K-W: .247	K-W: .765	-.043 .641	-.143 .094	-.292 .001	-.158 .064	-.095 .333
Haemoglobin	-.100 .224	K-W: .052	K-W: .102	K-W: .005	K-W: .823	K-W: .711	K-W: .026	.210 .020	K-W: .021	K-W: .704	.202 .024	- 200 .018	-.302 .000	-.190 .022	- 086 .316
MCV	.219 .009	K-W: .456	K-W: .851	K-W: .078	K-W: .095	K-W: .059	K-W: .069	-.166 .067	K-W: .914	K-W: .000	.284 .001	.119 .162	.205 .215	.199 .025	.084 .328
WCC	-.090 .340	K-W: .697	K-W: .011	K-W: .120	K-W: .000	K-W: .941	K-W: .402	-.434 .000	K-W: .473	K-W: .010	.285 .001	.460 .000	.470 .000	.452 .000	.292 .001
Platelets	-.026 .436	K-W: .195	K-W: .562	K-W: .419	K-W: .273	K-W: .211	K-W: .293	-.490 .000	K-W: .429	K-W: .037	.243 .007	.338 .000	.502 .000	.312 .000	.271 .001
ESR	.195 .030	K-W: .09	K-W: .613	K-W: .140	K-W: .595	K-W: .639	K-W: .746	.262 .000	K-W: .528	K-W: .010	-.053 .727	.246 .004	.361 .000	.291 .004	.149 .065

Notes: [illegible] As defined in Table 1.
[illegible] New or 'retreatment' patient as defined in Table 1.
[illegible] Formulation batches approved and not approved by the regulatory authority, as defined in Table 1.
[illegible] By chance, all HIV-infected patients received single-drug formulations.

Table 2b: Association matrix of plasma chemistry and haematology tests, displaying the statistical significance of the relationships.

p < 0.050

0.050 ≤ p < 0.100

---- Spearman's rank correlations were used if not otherwise specified; rho-value followed by p-value

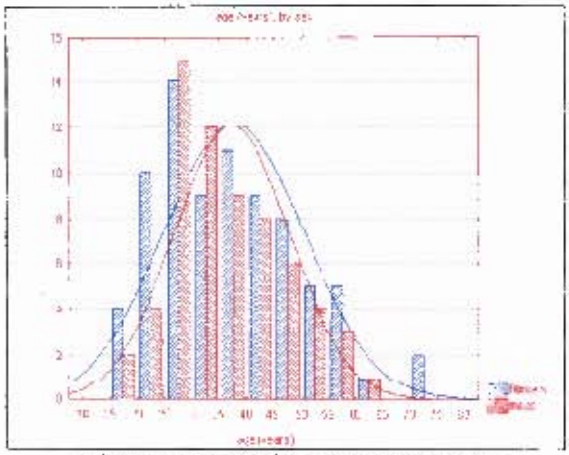
R Rifampicin

H Isoniazid

Due the large number of comparisons within the same patient sample it is likely that some of the weaker correlations are chance findings in this particular sample.

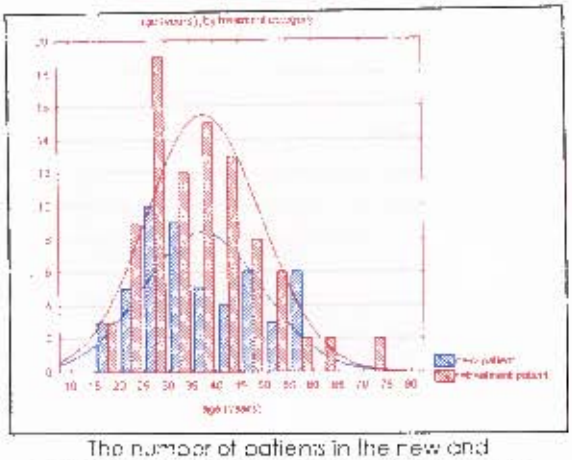
Covariate factors:	Total protein	Albumin	Total bilirubin	AST	ALT	AP	γ-GT	Urea	Creatinine	Haemoglobin	MCV	WCC	Platelets	ESR
Total protein														
Albumin	.324 .139													
Total bilirubin	.026 .757	.056 .807												
AST	.297 .000	.068 .513	.188 .224											
ALT	.045 .512	.210 .014	.094 .365	.729 .000										
AP	.121 .150	-.147 .079	.098 .348	.281 .011	.251 .006									
γ-GT	.206 .013	-.227 .006	.104 .314	.271 .001	.245 .002	.469 .000								
Urea	.064 .443	.176 .036	.032 .704	.120 .353	-.086 .324	-.030 .720	-.153 .085							
Creatinine	.019 .815	.198 .018	.057 .550	-.113 .181	-.004 .966	-.134 .113	-.035 .713	.417 .000						
Haemoglobin	.014 .866	.303 .000	.274 .001	.166 .047	.365 .000	-.060 .478	-.067 .461	-.144 .086	-.25 .34					
MCV	.155 .064	-.158 .025	.110 .192	.078 .356	-.064 .430	-.031 .715	.147 .078	.023 .785	-.018 .828	.133 .097				
WCC	.094 .290	-.207 .016	-.157 .061	-.053 .566	-.111 .184	.095 .350	.152 .069	-.127 .148	-.244 .002	.107 .238	.021 .811			
Platelets	.095 .324	-.262 .002	-.295 .000	-.142 .091	-.228 .006	.221 .009	.100 .299	-.127 .176	.218 .009	.096 .350	-.169 .053	.566 .000		
ESR	.355 .000	-.639 .000	-.249 .004	.040 .638	-.218 .010	.240 .005	.314 .000	.062 .498	.269 .002	.360 .000	.279 .001	.270 .001	.354 .000	

The distribution of patient ages was consistent with the nature of the tuberculosis patient population, with young and middle-aged adults comprising the majority. More females (55%) than males were enrolled in the study; especially amongst the youngest and oldest of the patients, but the distribution of the sexes was similar for most age groups.



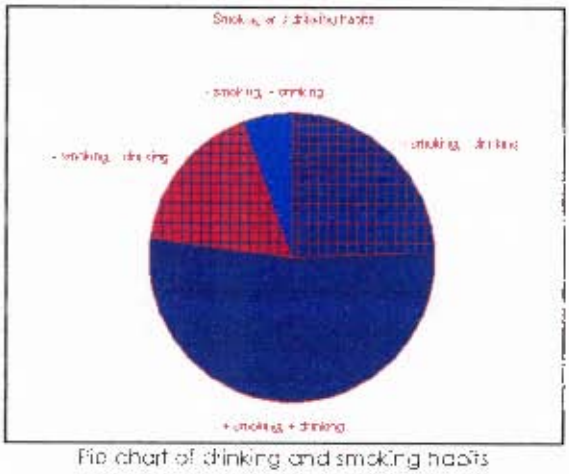
Histogram showing the distribution of age according to sex, in the study sample.

Most patients in the study population had received more than one month of antituberculosis treatment prior to their admission to Brewelskloof Hospital. Retreatment patients had lower body mass indices ($p = 0.038$), ALT values ($p = 0.000$) and AST values ($p = 0.033$), and higher doses of isoniazid ($p = 0.016$) and rifampicin ($p = 0.046$) for their weight than patients receiving antituberculosis treatment for the first time. The age distribution of patients receiving treatment for the first time was bimodal, with patients clustered in early and late adulthood.



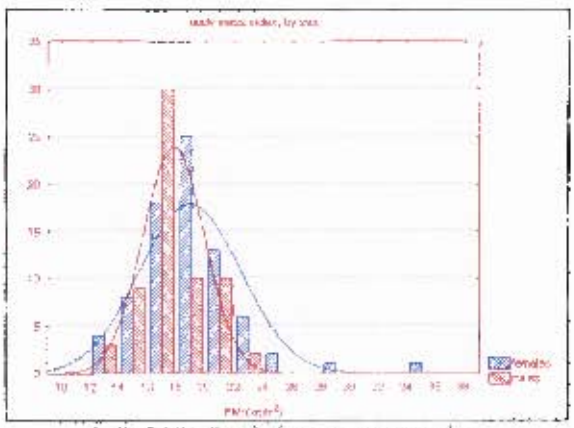
The number of patients in the new and retreatment categories, in different age groups.

The majority (53%) of the study patients smoked cigarettes and consumed alcohol regularly in the year prior to admission to the hospital. Those who neither smoked, nor drank alcohol regularly comprised 24%, whilst those who smoked but did not drink regularly accounted for 17% and those who drank alcohol regularly but did not smoke accounted for 6% of the study population.



Pie chart of drinking and smoking habits

Generally, patients had low body mass indices (BMIs). Retreatment patients, males ($p = 0.055$) and patients with markers of more severe illness (raised total protein, $p = 0.046$; low albumin $p = 0.000$; low haemoglobin, $p = 0.020$; raised white cell counts, $p = 0.000$; raised platelet counts, $p = 0.000$; and increased ESR, $p = 0.000$) had lower BMIs.

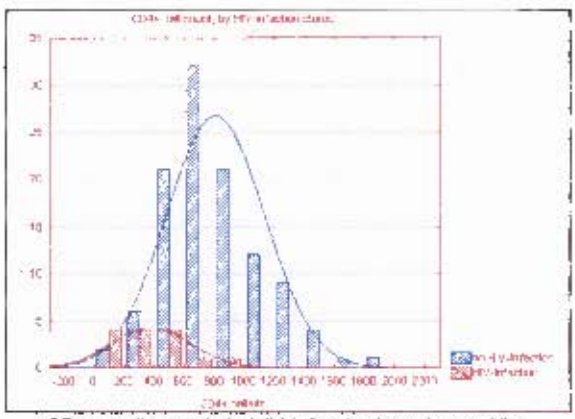


BMI distribution in females and males.

Lower BMIs were also correlated with higher drug doses per kilogram ($p = 0.000$). In addition, BMI was positively correlated with total bilirubin, ALT and urea, and negatively correlated with alkaline phosphatase and γ-GT.

Only 18% of the 93 patients with known acetylator type were slow metabolizers; in comparison to the 35% found by Parkin et al. in another Western Cape population (also with a predominantly mixed-race ancestry, but well enough to be treated as outpatients after an initial 2- to 3-day assessment period)[15]. Geographical variation, within the region, in the distribution of the genotypes could explain the difference. However, the possibility that intermediate and rapid acetylators are more likely to be hospitalized cannot be excluded.

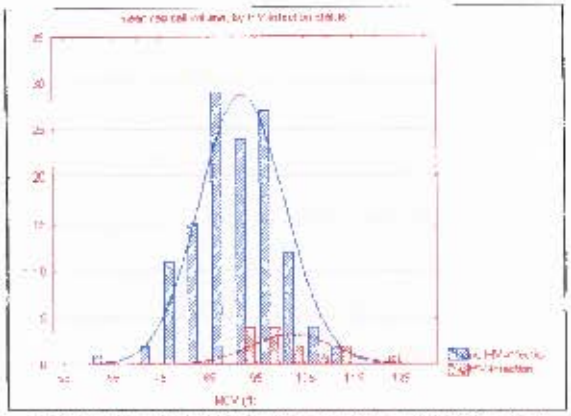
Antibodies to Human Immunodeficiency Virus were found in 10% of patients (7 females and 7 males). HIV-infection was correlated with non-smoker status ($p = 0.022$). More HIV-infected subjects were receiving treatment for the first time ($p = 0.021$), and (by chance) none received antituberculosis products containing rifampicin and isoniazid in combination ($p = 0.045$). No patient received antiretroviral treatment.



CD4+ cell counts in HIV-infected and non-HIV infected patients.

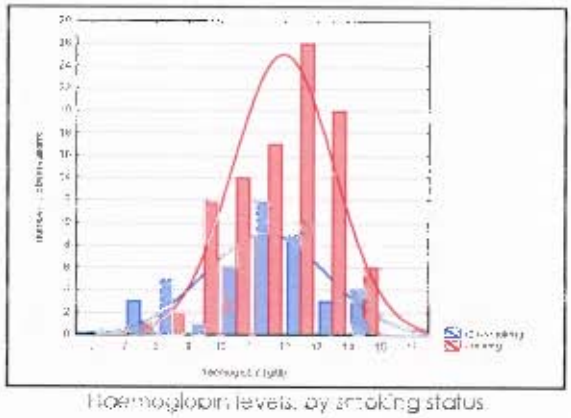
HIV-infected patients had lower CD4+ cell counts ($p = 0.003$), while cell counts ($p = 0.010$) and platelet counts ($p = 0.037$); and higher total protein ($p = 0.001$), ESR ($p = 0.010$) and mean cell volumes ($p = 0.000$). HIV-infection status was not correlated with BMI. HIV-

infected patients tended to receive slightly lower rifamycin doses per kilogram of body weight (10.23 mg/kg vs. 10.98 mg/kg, p = 0.068).



MCV values in HIV-infected and non-HIV infected patients.

Although no correlation was observed between smoking status and haemoglobin, non-smokers tended to be have a bimodal haemoglobin distribution. Perhaps, long-standing tuberculous lung disease was associated with a trend to increased haemoglobin in a group of patients. The effect seems to be obscured in smokers who are few amongst those with very low haemoglobin concentrations.



Haemoglobin levels, by smoking status

Drug doses (adjusted for patient weight) were higher in those with lower BMIs, and, correspondingly, tended to be correlated with indicators of more severe disease. Increased doses per kilogram of rifampicin, isoniazid, pyrazinamide and ethambutol were correlated with increased alkaline phosphatase and (barring ethambutol) decreased ALT levels. Increased doses per kilogram of rifampicin and pyrazinamide were associated with increased γ-GT levels. Almost all patients received a standard 300 mg dose of isoniazid; hence females had a higher dose per kilogram of body weight (p=0.004).

Fifty-four subjects (38%) received batches of single drug formulations of rifampicin that were not approved by the Medicines Control Council of South Africa, on the day of pharmacokinetic sampling. It is not known whether they received the non-approved batches for the duration of their hospital admission. Subsequent analysis demonstrated that lower rifampicin blood concentrations were achieved in patients who were dispensed the non-approved batches[28], and it is interesting to note that those patients who received the doses from the inferior batches on the day of PK sampling also tended to have lower BMIs, haemoglobin and bilirubin levels.

and higher platelet counts than those who received fully approved products. This possibly indicates that those who received the inferior batches were not responding to treatment as well as the other patients. However, the trends could also be related to the fact that patients weighing < 50 kg (with relatively low BMIs) were more likely to receive the non-approved batches (as the non-approved batches were more convenient for 450 mg doses than 600 mg doses).

A higher proportion of females (45%) than males (30%) received the non-approved rifampicin batches on the day of PK sampling (p=0.064). This can be explained by different ward stock for the male and female wards and differing distributions of the sexes in the weight categories used for dosing. For the same reasons, fixed dose combinations of rifampicin and isoniazid were received by 15% of females and 29% of males on the day of PK sampling (p=0.046).

Concomitant illness and medications in the study population are listed below (table 3). All subjects received rifampicin, isoniazid, and pyrazinamide, and 132 (93 %) received ethambutol. While several drug drug interactions with the antituberculosis agents could be anticipated, few would be expected to affect the concentrations of the antituberculosis agents.

Table 3: Concomitant illness and the use of concomitant medications in the study population

Concomitant drug	n
Amiloride / hydrochlorothiazide combination	2
Chlorpromazine	1
Co-trimoxazole	2
Digoxin	1
Ethionamide	2
$FeSO_4$	2
Fluphenazine	1
Folic acid	4
Furosemide	1
Glibenclamide	1
Gliclazide	2
Insulin	5
Magnesium trisilicate	1
Methyldopa	2
Orphenadrine	1
Perindopril	3
Phenobarbitone	1
phenytoin	2
pholcodeine	5
Piroxicam	2
Potassium chloride	1
Prednisone	2
Pyridoxine	124
Ranitidine	1
Streptomycin	68
Vit B co	61
Warfarin	1

Concomitant illness	n
Cardiomyopathy	1
Deep vein thrombosis	3
Diabetes mellitus	6
Epilepsy	2
HIV infection	4
Hypertension	5
Schizophrenia	1

THE RELATIONSHIP BETWEEN COVARIATES AND PHARMACOKINETIC MEASURES

Regression analyses were used to explore the influence of the patient and drug factors (independent variables) on the pharmacokinetic measures (Cmax, AUCt, AUCi, Tmax, lag-time and half-life and elimination rate constant) of rifampicin, isoniazid, pyrazinamide and ethambutol.

The covariate patient and drug factors entered into each regression included: age (in years), sex (0=females; 1=males), smoking within the year prior to admission (0=no; 1=yes), regular alcohol consumption in the year prior to admission (0=no; 1=yes), treatment category (0=not previously treated with antituberculosis drugs for > 1 month; 1=retreatment), HIV-infection (0=no; 1=yes), drug dose/kg (mg/kg), formulation type (0=single drug formulation; 1=FDC), formulations regulatory status (0=fully approved; 1=not approved), acetylator type (for isoniazid; 0=slow; 1=intermediate or fast), body mass index (kg/m^2), urea (mmol/l), creatinine(μmol/l), total protein (g/l), albumin (g/l), ALT (units/l), AST (units/l), γ-GT (units/l), total bilirubin(μmol/l), haemoglobin (g/dl), MCV (fl), WCC (cells x 10^9/l), platelet count (x 10^9/l), ESR (mm), CD4+ cells/mm^3. When the independent or dependent variables were dichotomized, it is described in the text.

To avoid decreases in the sample size analyzed in the regression analyses, and resulting loss of power, certain missing values were replaced as described below.

Age was not known for one female subject; the median age for that sex was inserted (median female age: 36 years). The body mass index (BMI) was missing for 21 subjects the median sex-specific BMI was used to replace the missing value (in males 17.64; in females 18.70). In this way the influence of sex and BMI on the model may have been underestimated, but there was greater power to detect the influence of the remaining variables.

The risk of spuriously significant results is increased when many analyses are performed on the same data set. The risk has been offset by the relatively strict significance levels used when selecting covariates.

RIFAMPICIN

Regression of covariate factors on rifampicin Cmax

In order to satisfy the assumptions of the multiple linear regression analysis, the Cmax values were transformed by taking their square root. A disadvantage of this transformation was that meaningful quantification of the effect of the covariate on Cmax was not possible. Although the contribution of total bilirubin to the model was significant at a level of only 0.055, it was included in the model as it made a significant contribution if potentially influential observations were dropped. Table 4 summarizes the linear regression equation describing the square root of Cmax. The model accounted for 28% of the variability observed for the square root of Cmax. Patients who received FDC formulations or non-approved batches of rifampicin capsules had lower peak concentrations of rifampicin. The presence of HIV-infection, male sex, lower haemoglobin concentrations and higher albumin levels were associated with decreased peak concentrations of the drug. Lower ALT, and higher AST levels correlated with increased rifampicin Cmax values.

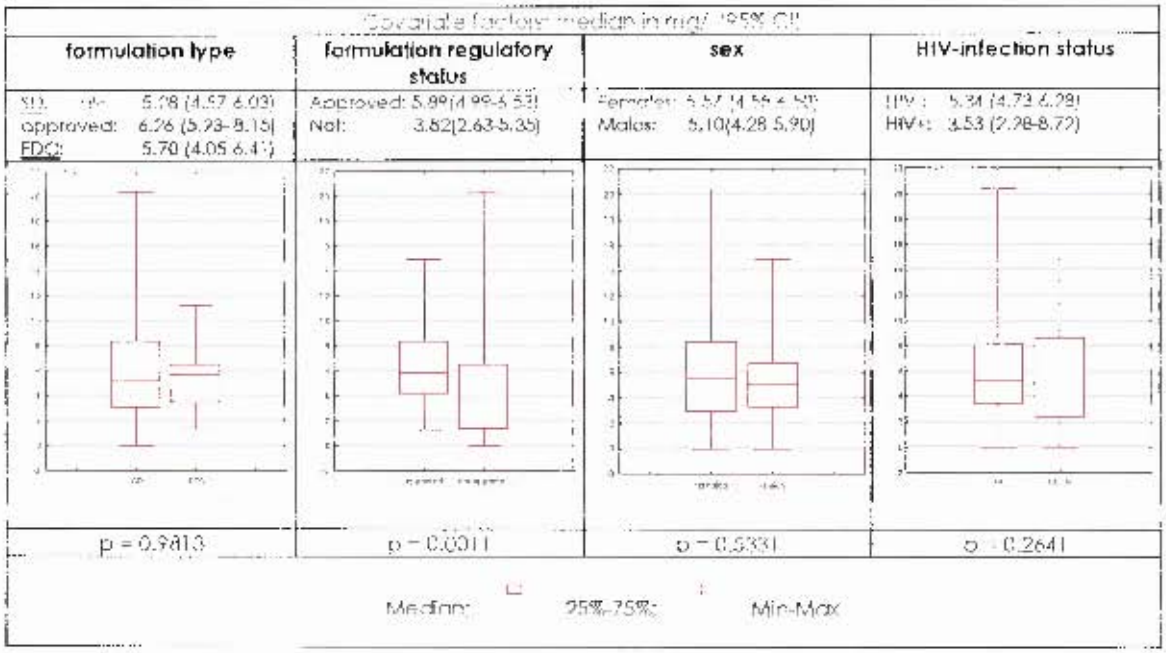
Table 4: Linear regression analysis of covariates on the square root of rifampicin Cmax.

Source	SS	Df	MS	Number of obs = 138	
Model	29.1307	9	3.2367	F(9, 128) = 5.72	
Residual	72.4472	128	0.5660	Prob > F = 0	
Total	101.5779	137	0.7414	R-squared = 0.2868	
				Adj R-squared = 0.2366	
Square root of Cmax				Root MSE = 0.7523	
	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]
Total bilirubin	0.0334	0.0173	1.94	0.055	0.0007 0.0676
ALT	-0.0225	0.0084	-2.69	0.008	-0.0391 -0.0059
AST	0.0342	0.0101	3.38	0.001	0.0142 0.0542
Albumin	-0.0367	0.0153	-2.4	0.018	-0.0671 -0.0064
haemoglobin	0.1331	0.0514	2.59	0.011	0.0315 0.2347
Sex	-0.3535	0.1420	-2.49	0.014	-0.6345 -0.0724
HIV-infection	-0.5249	0.2292	-2.29	0.024	-0.9783 -0.0715
Formulation type	-0.5076	0.1883	-2.7	0.008	-0.8801 -0.1351
Formulation regulatory status	-0.7705	0.1580	-4.88	0.000	-1.0831 -0.4578
Constant	2.0679	0.5605	3.69	0.000	0.9588 3.1770

The differences in the peak rifampicin levels by the formulation type (SD or FDC), the regulatory status (approved or non approved), sex and HIV-infection status are illustrated in figure 1. These levels are not adjusted for the other covariates; hence the median Cmax for FDCs is higher than that of single drug formulations when the non-approved products are included in the data set (i.e. there are 2 different effects within the single

drug formulation group: approved single drug products had greater bioavailability than FDCs, while the non-approved single drug products had reduced bioavailability).

Figure 1: Box-and-whisker plots of rifampicin Cmax by formulation characteristics, sex and HIV infection status. The statistical significance of the differences between the group medians was evaluated using the Kruskal-Wall's test.



Substitution of AST and ALT by the ratio of AST/ALT yielded a slightly stronger model (table 5), and explained better the relationship of the transaminases to the peak rifampicin concentration.

Table 5: Multiple linear regression model describing AST/ALT ratio and other covariates on the square root of Cmax

Source	SS	df	MS	Number of obs = 138
Model	29.3237	9	3.6655	F(8, 129) = 6.54
Residual	72.2542	129	.5601	Prob > F = 0
Total	101.5779	137	.7414	R-squared = 0.2887
				Adj R-squared = 0.2446
Sqrt(cmax)				Root MSE = .7484

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Total bilirubin	0.0303	0.0173	1.75	0.083	-0.0040	0.0646
AST/ALT ratio	0.5497	0.1559	3.53	0.001	0.2412	0.8582
Albumin	-0.0354	0.0153	-2.32	0.022	-0.0656	-0.0052
Haemoglobin	0.1585	0.0513	3.09	0.002	0.0570	0.2600
sex	-0.3639	0.1405	-2.59	0.011	-0.6418	-0.0860
HIV-infection	-0.4322	0.2195	-1.97	0.051	-0.8664	0.0021
Formulation type	-0.5132	0.1873	-2.74	0.007	-0.8837	-0.1426
Formulation regulatory status	-0.7455	0.1564	-4.77	0.000	-1.0549	-0.4361
constant	1.2986	0.6461	2.01	0.046	0.0205	2.5771

When the independent variables total bilirubin, AST, ALT, γ-GT and AP were dichotomized according to the normal ranges (values greater than the upper limit of the normal range

were assigned a value of 1, while values less than or equal to the upper limit of the range were assigned a value of 0). total bilirubin was found to contribute significantly to the model, but AST and ALT were not important (table 6). The other variables contributing to the model were the same and had similar coefficients to the model derived when continuous values were used for the liver function tests.

Table 6: Linear regression analysis of covariates on the square root of rifampicin Cmax; dichotomized total bilirubin, AST, ALT, γ-GT and AP.

Source	SS	df	MS	Number of obs = 138
Model	20.9255	7	2.9893	F(7, 130) = 4.82
Residual	80.6526	130	0.6204	Prob > F = 0.0001
Total	101.5779	137	0.7414	R-squared = 0.206
				Adj R-squared = 0.1632
Square root of Cmax				Root MSE = 0.78757

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
HIV-infection	-0.4247	0.2312	-1.84	0.069	-0.8822	0.0328
Albumin	-0.0344	0.0163	-2.11	0.036	-0.0665	-0.0022
Total bilirubin (<18=0; >17=1)	0.9101	0.4723	1.93	0.056	-0.0242	1.8445
Haemoglobin	0.1185	0.0520	2.28	0.024	0.0155	0.2214
Sex	-0.3217	0.1481	-2.17	0.032	-0.6148	-0.0287
Formulation type	-0.4495	0.1955	2.30	0.023	-0.8363	-0.0627
Formulation regulatory status	-0.7437	0.1620	-4.59	0.000	-1.0642	-0.4231
Constant	2.5992	0.5682	4.57	0.000	1.4751	3.7233

Logistic regression analyses were used to assess the risk attributed to covariate factors of low rifampicin peak concentrations (< 4 mg/l: 50% below the reference range often quoted in the literature[56,57]) and very low rifampicin peak levels (< 2 mg/l). The results are shown in tables 7 and 8, respectively.

Table 7: Logistic regression analysis of covariates on Cmax to determine the risk of low rifampicin peak concentrations

Logistic regression					Number of obs = 138	
Log likelihood = -70.4048					LR chi2(5) = 34.87	
					Prob > chi2 = 0	
Cmax < 4 mg/l					Pseudo R2 = 0.1985	
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
Formulation type	4.0437	2.5362	2.23	0.026	1.1828	13.8246
Formulation regulatory status	11.2463	6.2119	4.38	0.000	3.8093	33.2027
HIV infection	5.1808	6.2706	2.74	0.006	1.8211	36.7493
ALT	1.0944	0.0343	2.88	0.004	1.0292	1.1637
AST	0.8471	0.0439	-3.2	0.001	0.7653	0.9376

Patients receiving rifampicin-containing products not fully registered with the regulatory authority were 11.25 times more likely, than those who received fully approved products, to have Cmax values <4 mg/l. HIV-infection was associated with an 8.18 times relative risk for Cmax < 4mg/l. When ALT and AST were dichotomized as described above into values below the upper limit of the normal range and values above this range, they did

not contribute to the model significantly. However, when AST and ALT were entered as continuous variables, there was a 9 % increased risk of Cmax < 4mg/l associated with each unit increase of ALT. Conversely, each unit increase in AST was associated with 15 % reduction in the risk of having a Cmax < 4 mg/l.

Table 8: Logistic regression analysis of covariates on Cmax to determine the risk of VERY low rifampicin peak concentrations

Logistic regression Log likelihood = -33.4375 **Cmax < 2 mg/l**					Number of obs = 141 LR chi2(5) = 44.61 Prob > chi2 = 0 Pseudo R2 = 0.4001	
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. interval]	
Formulation regulatory status	30.0268	27.2547	3.75	0.000	5.0687	177.8795
HIV-infection	9.9857	11.0151	2.09	0.037	1.1493	86.7614
Age	0.9346	0.0330	-2.11	0.035	0.8777	0.9952
AST	0.8467	0.0549	-2.57	0.010	0.7457	0.9614
Total bilirubin	0.7389	0.1079	2.07	0.038	0.5550	0.9837

Assuming approximately 80 - 90% of rifampicin in plasma is protein bound, the free drug concentration of 0.2 – 0.4 mg/l corresponds to a total drug concentration of 2 mg/l, and this range approximates the MIC of susceptible *M. tuberculosis* (0.25 to 0.50 mg/l) to rifampicin. The relative risk of a Cmax < 2 mg/l was increased 30.03 times if an unregistered drug product was taken on the day of pharmacokinetic assessment. HIV-infection was also associated with a much greater risk of a very low Cmax: 9.99 times that in patients without HIV-infection. Each unit increase in AST and total bilirubin were associated with risk reductions of 15% and 26% respectively, for a Cmax ≥ 2 mg/l. Older patients were less likely to have a very low Cmax, with a risk reduction of 7% for each year.

Regression of covariate factors on rifampicin AUCt

The square root of the dependent variable was used in a multiple linear regression analysis to determine those covariate factors that influenced the AUCt significantly (Table 9). The model accounted for 34% of the variability displayed in the square root of AUCt. As was shown for the square root of Cmax, the formulation type and regulatory status were important determinants of the AUCt (FDC formulations or the non-approved batches of rifampicin capsules were associated with lower peak concentrations of rifampicin). The presence of HIV-infection, male sex, lower haemoglobin, higher albumin, higher ALT, lower AST and lower bilirubin levels were again associated with decreased drug concentrations as measured by the AUCt.

Table 9: Multiple linear regression of covariates on AUCt with square root transformation of the dependent variable

Source	SS	DF	MS	Number of obs = 137
Model	140.832715	9	15.6480	F(9, 127) = 7.16
Residual	277.6794	127	2.1865	Prob > F = 0
Total	418.5009	136	3.0772	R-squared = 0.3365
				Adj R-squared = 0.2895
Square root of AUCt				Root MSE = 1.4787

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
HIV-infection	-1.0837	0.4504	-2.41	0.018	-1.9750	-0.1925
Albumin	-0.0923	0.0301	-3.06	0.003	-0.1519	-0.0327
Total bilirubin	0.0856	0.0339	2.52	0.013	0.0184	0.1527
ALT	-0.0529	0.0165	-3.22	0.002	-0.0855	-0.0204
AST	0.0720	0.0199	3.62	0.000	0.0326	0.1113
Haemoglobin	0.2872	0.1010	2.84	0.005	0.0874	0.4870
Sex	-0.7433	0.2811	-2.64	0.009	-1.2996	-0.1871
Formulation type	-1.0778	0.3749	-2.87	0.005	-1.8198	-0.3359
Formulation regulatory status	-1.6301	0.3106	-5.25	0.000	-2.2448	-1.0154
Constant	4.3509	1.1017	3.95	0.000	2.1702	6.5303

When the AST/ALT ratio replaced AST and ALT in the model a stronger relationship between the independent variables and the square root of AUCt emerged (table 10).

Table 10: Multiple linear regression model describing AST/ALT ratio and other covariates on the square root of AUCt

Source	SS	df	MS	Number of obs = 137
Model	141.6356	8	17.7048	F(8, 128) = 8.19
Residual	276.8625	128	2.1630	Prob > F = 0
Total	418.5009	136	3.0772	R-squared = 0.3384
				Adj R-squared = 0.2971
Sqrt(AUCt)				Root MSE = 1.4707

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
Total bilirubin	0.0790	0.0341	2.32	0.022	0.0116	0.1464
AST/ALT ratio	1.1934	0.3068	3.89	0.000	0.5862	1.8005
Albumin	-0.0895	0.0300	-2.98	0.003	-0.1488	-0.0302
Haemoglobin	0.3329	0.1008	3.30	0.001	0.1334	0.5323
Sex	-0.7460	0.2781	-2.68	0.008	-1.2963	-0.1958
HIV-infection	-0.9261	0.4313	-2.15	0.034	-1.7795	-0.0727
Formulation type	-1.0727	0.3726	-2.88	0.005	-1.8101	-0.3354
Formulation regulatory status	-1.5744	0.3074	-5.12	0.000	-2.1826	-0.9661
constant	2.6771	1.2700	2.11	0.037	0.1642	5.1900

As for the model describing Cmax, when the independent variables total bilirubin, AST, ALT, γ-GT and AP were dichotomized according to the normal ranges, AST, ALT, γ-GT and AP did not contribute significantly to the model describing AUCt (Table 11).

Table 11: Multiple linear regression of covariates on the square root of AUCI using dichotomized variables for total bilirubin, AST, ALT, γ-GT and AP

Source	SS	Df	MS	Number of obs = 137
Model	99.7632	7	14.2519	F(7, 1291) = 5.77
Residual	318.7377	129	2.4705	Prob > F = 0
Total	418.5009	136	3.0772	R-squared = 0.2384
				Adj R-squared = 0.1971
Square root of AUCI				Root MSE = 1.5719

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	interval]
HIV-infection	-0.9181	0.4615	-1.99	0.049	-1.8311	-0.0050
Albumin	-0.0863	0.0324	-2.66	0.009	-0.1505	-0.0222
Total bilirubin [<18=0; >17=1]	2.2212	0.9425	2.36	0.020	0.3565	4.0859
Haemoglobin	0.2487	0.1039	2.39	0.018	0.0431	0.4542
Sex	-0.6533	0.2979	-2.19	0.030	-1.2426	-0.0640
Formulation type	0.9526	0.3955	2.41	0.017	1.7351	0.1700
Formulation regulatory status	-1.5860	0.3235	-4.90	0.000	-2.2261	-0.9460
Constant	5.5196	1.1340	4.87	0.000	3.2760	7.7631

Regression of covariate factors on Rifampicin AUCI

To satisfy the assumptions of multiple linear regression analysis, the natural logarithm of the dependent variable was used to determine the impact of covariate factors. The model tabulated below (table 12) explains 26% of the variability in ln(AUCi).

Table 12: Multiple linear regression of the covariate factors on the log transformed AUCi

Source	SS	Df	MS	Number of obs = 124
Model	14.0097	6	2.3366	F(6, 117) = 6.82
Residual	40.1222	117	0.3429	Prob > F = 0
Total	54.1319	123	0.4403	R-squared = 0.2591
				Adj R-squared = 0.2211
ln(AUCi)				Root MSE = 0.5856

	Coef	Std. Err.	T	P>|T|	[95% Conf.	Interval]	effect of 1 unit increase on AUC
HIV-infection	-0.3277	0.1833	-1.79	0.076	-0.6907	0.0353	↓28%
Total bilirubin	0.0506	0.0135	3.91	0.000	0.0243	0.0769	↑5%
ALT	-0.0213	0.0064	-3.34	0.001	-0.0339	-0.0087	↓2%
AST	0.0247	0.0079	3.12	0.002	0.0090	0.0404	↑3%
Formulation regulatory status	-0.2721	0.1164	-2.34	0.021	-0.5027	-0.0415	**↓24%**
Sex	-0.1426	0.1095	-1.30	0.195	-0.3594	0.0742	**↓13%**
Constant	2.8733	0.1682	17.08	0.000	2.5406	3.2070	

Although p>0.05 for sex, the variable is included in the model because when potentially influential observations were removed, it contributed significantly; it is therefore a significant determinant for a model including most (non-outlying) observations. Non approved products were associated with a 24% reduction in the AUCi. HIV infection was associated with a reduction of 28% in the AUCi although the association was not significant at the 0.05 level (p = 0.076). Unit increases in total bilirubin and AST were associated with 5% and 3% increases in the AUCi, respectively. Each 1 unit decrease in

ALT was associated with an increase of 2% in the AUCi. Formulation type was not significant in the model describing ln(AUCi); albumin and haemoglobin were also non-contributory.

As for the models describing Cmax and AUCt, a stronger model was generated when the AST/ALT ratio replaced AST and ALT in the model (table 13).

Table 13: Multiple linear regression model describing AST/ALT ratio and other covariates on ln(AUCi)

Source	SS	df	MS	Number of obs = 124
Model	14.0691	5	2.8538	F(5, 118) = 5.44
Residual	39.8528	118	.3380	Prob > F = 0
Total	54.1519	123	.4403	R-squared = 0.2635
				Adj R-squared = 0.2323
ln(AUCi)				Root MSE = 0.5814

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
HIV-infection	-0.3217	0.1778	-1.81	0.073	-0.6737	0.0305
Total bilirubin	0.0492	0.0132	3.72	0.000	0.0230	0.0754
AST/ALT ratio	0.4278	0.1147	3.73	0.000	0.2006	0.6550
Formulation regulatory status	-0.2426	0.1151	-2.28	0.024	-0.4906	-0.0346
Sex	-0.1237	0.1091	1.13	0.259	-0.3899	0.0924
Constant	2.4365	0.1854	13.14	0.000	2.0693	2.8037

Regression of covariate factors on Rifampicin elimination rate constant (ke)

Logarithmic transformation of Ke was used in the multiple linear regression analysis shown below (table 14):

Table 14: Multiple linear regression model of covariates on log-transformed elimination rate constant

Source	SS	df	MS	Number of obs=118
Model	5.9614	8	.7452	F(8 109)=10.27
Residual	7.9107	109	.0726	Prob > F=0
Total	13.8721	117	.1186	R-squared=0.4297
				Adj R-squared=0.3879
Ln(ke)				Root MSE=0.2694

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]		% change in ke for 1 unit increase
ESR	-0.0040	0.0008	-5.35	0.000	-0.0055	-0.0025	↓0.40%
HIV-infection	0.1815	0.0972	1.87	0.064	-0.0111	0.3741	↑18%
Age	-0.0054	0.0023	-2.32	0.022	-0.0100	-0.0008	↓0.53%
Smoking	0.1169	0.0565	2.07	0.041	0.0050	0.2288	↑11.69%
Total bilirubin	-0.0350	0.0065	-5.39	0.000	-0.0478	-0.0221	↓3.50%
Creatinine	-0.0067	0.0016	-4.24	0.000	-0.0098	-0.0036	↓0.67%
Mean cell volume	0.0069	0.0032	2.15	0.034	0.0005	0.0133	↑0.69%
White cell count	0.0150	0.0082	1.85	0.071	0.0012	0.0313	↑1.50%
constant	-0.4786	0.3021	-1.58	0.116	-1.0774	0.1202	

The model explained 43% of the variability in ln(ke). HIV-infected patients and those who smoked in the year prior to admission had increased clearance, reflected by 18% and 12% increases in Ke, respectively. Higher total bilirubin levels were associated with

decreased rifampicin clearance) for each unit increase in total bilirubin, a 3.5% reduction was seen in ke. Higher MCV values were associated with minor increases in ke, and increased age, creatinine, white cell count and ESR were associated with decreased rifampicin elimination.

Regression of covariate factors on Rifampicin half-life

Similarly, the natural logarithm of the half-life was used in a multiple linear regression analysis. The model is summarized in table 15. As expected, the same covariates influenced the model for ln(half life) as for ln(ke), but in an inverse manner.

Table 15: Multiple linear regress on model of covariates inc log-transformed half-life of rifampicin

Source	SS	df	MS	Number of obs=118
Model	5.7619	[illegible]	[illegible]	[illegible]
Residual	7.9116	109	.0726	Prob > F=0
Total	13.6735	117	.1 86	R-squared=0.4297
				Adj R-squared=0.3879
Ln(half-life)				Root MSE=0.2694

	Coef.	Std. Err.	t	P>|t|	[95% Conf. interval]		% change in ke for 1 unit increase
ESR	0.0040	0.0008	5.35	0.000	0.0025	0.0055	↑0.40%
HIV-infection	-0.1815	0.0972	-1.87	0.064	-0.3741	0.0111	↓18%
Age	0.0054	0.0023	2.32	0.022	0.0008	0.0100	↑0.54%
Smoking	0.1169	0.0565	2.07	0.041	0.2289	0.0050	↓11.69%
Total bilirubin	0.0350	0.0065	5.39	0.000	0.0221	0.0478	↑3.50%
Creatinine	0.0067	0.0016	4.24	0.000	0.0036	0.0098	↑0.67%
Mean cell volume	-0.0069	0.0032	-2.16	0.034	-0.0134	-0.0005	↓0.69%
White cell count	0.0150	0.0082	1.83	0.071	-0.0013	0.0312	↑1.50%
constant	-0.4786	0.3021	-1.58	0.116	-1.0774	0.1202	

Regression of covariate factors on the time for rifampicin to reach maximum concentration

The natural logarithm of Tmax was used in the model described in table 16. Delayed peak systemic concentrations were associated with non-approved products, FDCs, patients on retreatment regimens, HIV-infection, elevated white cell counts, total protein and urea. Higher ESR values and haemoglobin levels were associated with more rapid attainment of peak concentrations.

The risk of delayed absorption (a nonzero lag-time)

The assumptions of the linear regression model could not be satisfied using the continuous variable lag-time, the natural logarithm or the square root of the lag time.

lag time was therefore dichotomized. A logistic regression model (table 17) was used to determine the risk of a lag-time > 0 associated with the covariates. The use of non-approved products was associated with a 2.69 times greater risk of a nonzero lag-time compared to approved products. Each unit increase in urea was associated with a 56% increased risk of a nonzero lag-time. Increased age and haemoglobin were associated with a decreased risks of a nonzero lag-time (7% and 32% reductions, respectively, for each unit).

Table 16: Multiple linear regression model of the covariate factors on the log transformed PK measure Tmax

Source	SS	df	MS	Number of obs = 130
Model	6.4732	9	0.7192	F(9, 120) = 5.18
Residual	16.6701	120	0.1389	Prob > F = 0.000
Total	23.1434	129	0.1794	R-squared = 0.2797
				Adj R-squared = 0.2257
Ln(tmax)				Root MSE = 0.3727

	Coef.	Std. Err.	T	P>|t|	[95% Conf.	Interval]
Treatment category	0.1772	0.0702	2.53	0.013	0.0383	0.3162
HIV-infection status	0.2998	0.1279	2.34	0.021	0.0466	0.5530
White cell count	0.0297	0.0104	2.86	0.005	0.0092	0.0502
Total protein	0.0157	0.0046	3.27	0.001	0.0062	0.0252
Urea	0.0699	0.0251	2.79	0.006	0.0203	0.1196
ESR	-0.0045	0.0012	-3.60	0.000	-0.0070	-0.0020
Formulation type	0.3621	0.0953	3.80	0.000	0.1734	0.5508
Formulation regulatory status	0.1973	0.0754	2.62	0.010	0.0481	0.3464
haemoglobin	-0.0608	0.0266	-2.28	0.024	-0.1135	-0.0081
Constant	-0.2238	0.4606	0.49	0.628	-1.1360	0.6885

Table 17: Logistic regression model, showing the relative risks of delayed absorption (a measurable lag time) associated with the covariate factors

Logit estimates	Number of obs = 142
Log likelihood = -73.6247	LR chi2(4) = 37.78
	Prob > chi2 = 0
Lag-time > 0	Pseudo R2 = 0.2038

	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Age	0.9289	0.0186	3.68	0.000	0.8932	0.9661
Formulation regulatory status	2.6907	1.0991	2.42	0.015	1.2083	5.9917
Haemoglobin	0.6840	0.0899	-2.89	0.004	0.5286	0.8851
Urea	1.5645	0.2937	2.38	0.017	1.0829	2.2602

Discussion of the determinants of rifampicin PK measures

The patient and treatment factor predictors of the PK measures for rifampicin are summarized in table 18.

Table 16: Summary of the covariate factors influencing rifampicin pharmacokinetic measures, as determined by the regression analyses. Those variables contributing to the final models are indicated by ↑ (an increase in the relevant variable was associated with an increase in the value of the PK measure), or ↓ (a decrease in the relevant variable was associated with a smaller value for the PK measure); the p-values of weak associations (p>0.05) are indicated in brackets. Multiple linear regression was used for all dependent variables except Tlag, for which logistic regression analysis was used after dichotomizing the variable.

Rifampicin							
PK measure	Cmax	AUC^{0-t}	AUC$^{0-\infty}$	Ke	t1/2	Tmax	Tlag
Transformation	sqrt	Sqrt	ln	ln	ln	ln	0=0hr/1=>0hr
R-squared for model	0.29	0.34	0.26	0.43	0.43	0.28	0.20 (Pseudo R²)
Covariate factors:							
Age (years)	↔	↔	↔	↓	↑	↔	↓
Sex (female=0; male=1)	↓	↓	↓ (0.058)	↔	↔	↔	↔
Smoking[1] (no=0, yes=1)	↔	↔	↔	↑	↓	↔	↔
Alcohol[2] (no=0, yes=1)	↔	↔	↔	↔	↔	↔	↔
Treatment category[3] ('new'=0; 'retreatment'=1)	↔	↔	↔	↔	↔	↑	↔
Formulation type[4] (SD=0; FDC=1)	↓	↓	↔	↔	↔	↑	↔
Formulation regulatory status[5] (approved=0; not approved=1)	↓	↓	↓	↔	↔	↑	↑
Rifampicin dose/kg	↔	↔	↔	↔	↔	↔	↔
BMI (kg/m²)	↔	↔	↔	↔	↔	↔	↔
Total protein (g/l)	↔	↔	↔	↔	↔	↑	↔
Albumin (g/l)	↓	↓	↔	↔	↔	↔	↔
Total bilirubin (µmol/l)	↑ (0.055)	↑	↑	↓	↑	↔	↔
AST (units/l)	↑	↑	↑	↔	↔	↔	↔
ALT (units/l)	↓	↓	↓	↔	↔	↔	↔
AP (units/l)	↔	↔	↔	↔	↔	↔	↔
γ-GT (units/l)	↔	↔	↔	↔	↔	↔	↔
Urea (mmol/l)	↔	↔	↔	↔	↔	↑	↑
Creatinine (µmol/l)	↔	↔	↔	↓	↑	↔	↔
Haemoglobin (g/dl)	↑	↑	↔	↔	↔	↓	↓
MCV (fl)	↔	↔	↔	↑	↓	↔	↔
WCC (10⁹/l)	↔	↔	↔	↓	↑	↑	
Platelets (10⁹/l)	↔	↔	↔	↔	↔	↔	↔
ESR (mm)	↔	↔	↔	↓	↑	↓	↔
HIV antibodies (Elisa negative=0; positive=1)	↓	↓	↓ (0.064)	↑ (0.064)	↓ (0.064)	↑	↔
CD4 (cells/ml)	↔	↔	↔	↔	↔	↔	↔

↔: no significant contribution to the regression model describing the PK measure

[1] history of smoking in the year prior to admission to Brewelskloof Hospital.

[2] history of regular alcohol consumption in the year prior to admission to Brewelskloof Hospital

[3] patients in 'new' category had not received more than 1 month of drug treatment prior to admission; patients in the 'retreatment' category had previously received anti-tuberculosis drugs for one month or longer.

[4] SD: Single drug formulations were used; Tablets or capsules each containing a single drug were administered together.

FDC: Rifampicin-containing fixed dose combination formulations were used. Rifampicin was combined with isoniazid in Rifinah® (rifampicin 300 mg with isoniazid 150 mg or rifampicin 150 mg with isoniazid 75 mg); single drug formulations of pyrazinamide and ethambutol were used.

[5] 54 subjects received a single drug rifampicin formulation that was not approved by the regulatory authority on the day of PK profile determination[6].

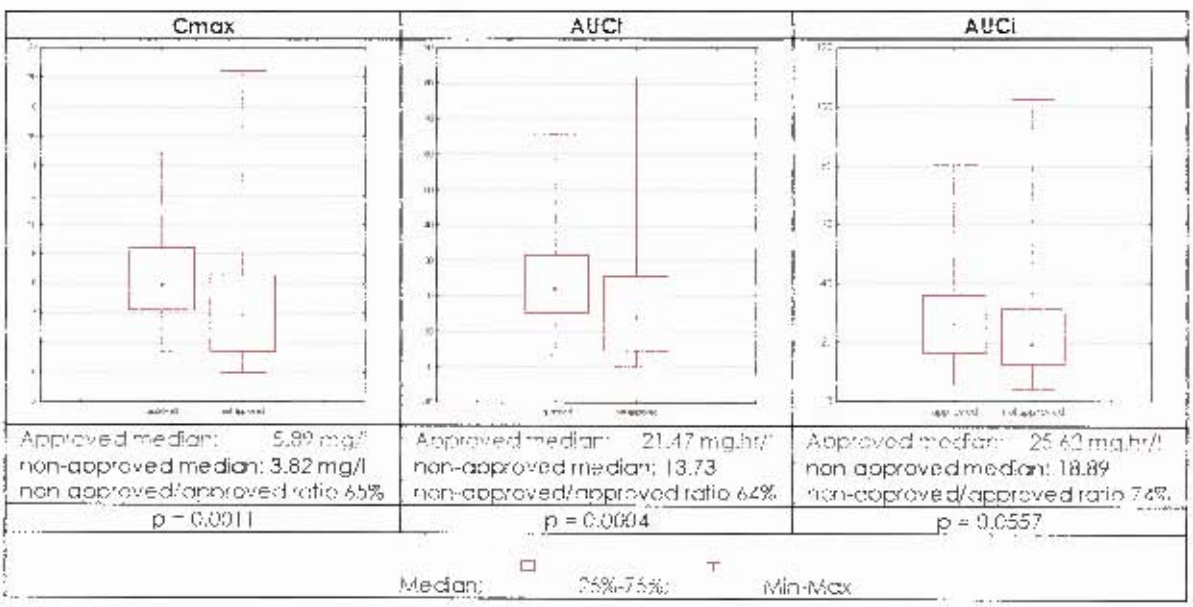
The important factors associated with reduced rifampicin bioavailability, as reflected by decreased Cmax, AUCt and AUCi values, were the use of the non-approved rifampicin product batches, the use of fixed dose combinations (rifampicin formulated together with isoniazid), male sex and HIV-infection.

Formulation is recognized to be an important determinant of rifampicin absorption[32,33,75,97]. The quality of the raw rifampicin, the excipients added and the conditions of heat and humidity during the manufacturing process[96], are thought to be important. That *in vivo* bioequivalence testing of rifampicin-containing FDCs is required prior to their use in patients is generally accepted[28,31,33]. Furthermore, exposure of FDC products to heat, humidity and light was shown by Singh et al. to accelerate an interaction of rifampicin and isoniazid forming the decomposition product isonicotinyl hydrazone of 3 formylrifamycin and isoniazid[97]. Dramatic reductions in the rifampicin content and lesser reductions in isoniazid content were demonstrated with exposure of the products to 75% relative humidity at 40°C for 12 days. Thus the packaging and storage conditions of products having rifampicin and isoniazid in combination is important. The FDC products used by the patients in this study had undergone and passed bioequivalence testing before registration with the national regulatory authority. The reduced bioavailability demonstrated here is therefore of concern. Excluding the data from those who received formulations not approved by the regulatory authority, 9% and 13% reductions in the median Cmax and AUCt, respectively, and a 25% increase in the median Tmax, were observed for FDC products without adjustment for the other covariates (p=0.0949, p=0.1016 and p=0.0025, respectively for Cmax, AUCt and Tmax). It is not known whether the relatively lower Cmax and AUCt values determined in these patients is related to deterioration whilst on the shelf (although no products were expired) resulting in reduced bioavailability, or to inferior batch quality (in comparison to the batches that underwent bioequivalence testing before product registration), or to variations in the bioavailability of the comparator products (in this study and the bioequivalence studies), or to disease-product interactions (such that bioequivalence testing procedures in healthy volunteers are not adequate to assure bioavailability in patients). The median time to expiry of the products used on the day of PK sampling was 17 months, with a range of 3 to 42 months. FDC products had a longer time to expiry (median of 39 months) than single drug products (median time to expiry of 14 months). Moreover, time to expiry did not contribute significantly to the regression models

describing lag time and AUCi, when it was added as a covariate. Multi-colinearity (a high degree of correlation between time to expiry and the formulation type; p=0.000) prevented an analysis of its effect on the regression models describing Tmax, Cmax and AUCt.

The findings in this study that the non approved product batches had inferior bioavailability (figure 2), and that non-approved batches were associated with a greatly increased risk of very low drug concentrations (table 8), support the necessity of *in vivo* bioequivalence testing of rifampicin-containing products (including single-drug formulations and even after only minor changes in the manufacturing process).

Figure 2: Box-and-whisker plots illustrating the differences in Cmax, AUCt and AUCi for those receiving approved and non-approved (right-hand side) batches. The statistical significance of the differences was evaluated using the Kruskal-Wallis test.



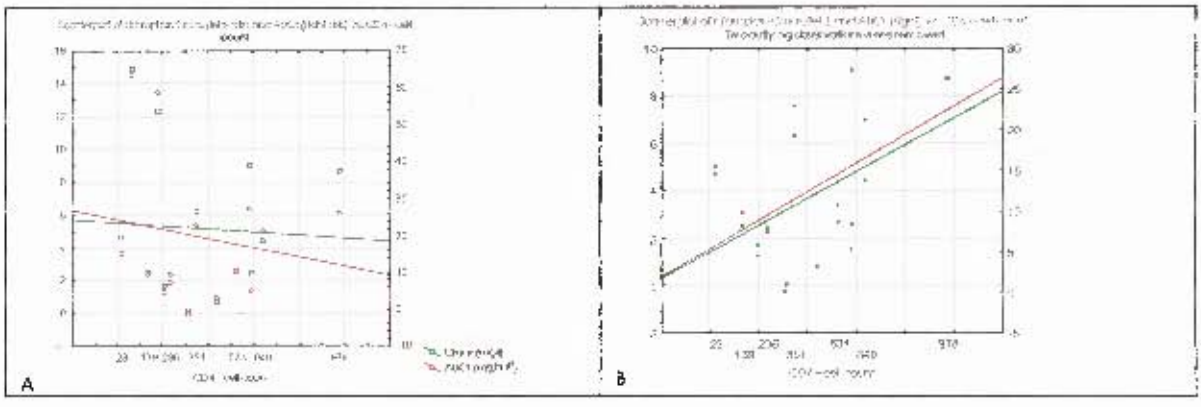
Slower dissolution rates might explain the delayed peak concentrations associated with use of the FDCs and non-approved products. Use of the non-approved products was also associated with an increased risk of a nonzero lag-time.

The association of HIV infection with reduced antituberculosis drug bioavailability has been reported previously[52,53,56,69]. It seems to be associated to some extent with the severity of HIV-infection and diarrhoea[69]. A minority of patients in this study had HIV-infection, and the variability of the Cmax and AUC values was such that multiple regression analysis was needed to adjust for other factors causing variability, in order for

the association to be recognized as significant. None-the-less, HIV-infection was associated with reduced Cmax, AUCt and AUCi values; with 34%, 36% and 16% reductions in the median values, respectively. Furthermore, the risk of very low (< 2 mg/l) rifampicin peak concentrations was 90% greater than that for the rest of the study population (table 8). These findings are similar to those of Sahai et al.[69] who found 41% and 32% reductions in the Cmax and AUC of rifampicin, respectively, in HIV-infected volunteers without tuberculosis in comparison to healthy volunteer controls. The greater reductions in the PK measures observed by Sahai et al. might be attributed to relatively advanced HIV-infection (2/3 of the HIV-infected group had CD4+ cell counts < 200), the presence of diarrhoea in 1/3 of the HIV-infected group and the absence of tuberculosis in the control group.

Sahai et al. found that rifampicin concentrations tended to be more markedly reduced in those with more advanced HIV-infection. A similar trend could be observed amongst the HIV-infected patients in this study (figure 3) when 2 outlying observations were removed, but the number of patients (14) was too small to establish the significance of the relationship between the stage of HIV-infection and rifampicin levels.

Figure 3: The relationship between CD4+cell count, and Cmax and AUCt, respectively, in all HIV-infected patients (A; n=14), and after removal of 2 outlying observations (B; n=12).



The time to reach Cmax was prolonged in HIV-infection (a finding in agreement with the trend observed by Sahai et al.), indicating that systemic absorption of the drug was not just reduced in those patients but also delayed. Increased expression of P glycoprotein at some sites has been demonstrated in the presence of HIV-infection[105]. If HIV-infection causes increased intestinal P glycoprotein expression, it could be implicated in both reduced absorption and delayed peak concentrations. Furthermore, HIV-infection was

associated with more rapid rifampicin elimination in this study. This is also consistent with increased p-glycoprotein expression, as the transmembrane efflux pump is also found on the canalicular surface of hepatocytes[75]. Although the differences in the half-lives of rifampicin between the groups in Sahai's study were not significant, asymptomatic HIV infected individuals with relatively early disease had a shorter mean half-life than the healthy controls, while those with CD4+ cell counts below 200 tended to have less efficient drug elimination than the control group.

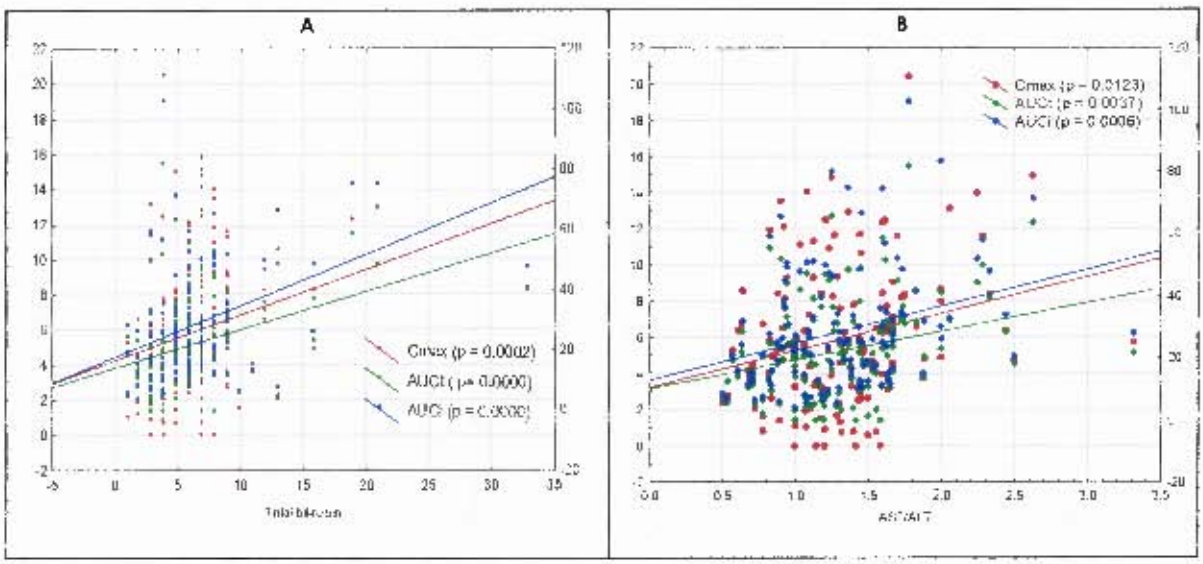
Males had reduced Cmax and AUC values independent of rifampicin dose/kg, a finding supported by that of Van Crevel et al.[75] in Indonesian patients. The reason for this is not clear: the drug dose/kg did not contribute to the regression models describing these measures, indicating that it was poorly associated with rifampicin concentrations; male sex was not significantly associated with delayed absorption or enhanced elimination rate; and BMI (a marker of body composition) was not associated with altered bioavailability. Besides, the superior distribution of rifampicin to fat in comparison to muscle[39] would in any event tend to lower the drug's concentrations in women more than in men.

Although the dose of rifampicin ranged from 8.82 – 14.20 mg/kg, the dose of rifampicin per kilogram of body weight was not an important determinant of the Cmax or AUC, even when adjusted for the other covariate factors. The lack of association highlights the intrinsic variability in the bioavailability of rifampicin, and supports the need to investigate factors that do have an impact on drug concentrations in patients.

The increased Cmax, AUC and prolonged half-life of rifampicin, in association with elevated bilirubin levels is consistent with the competition of the drug with bilirubin for biliary elimination[39]. The finding could also reflect reduced hepatic capacity to clear bilirubin and rifampicin. The transaminases AST and ALT were associated with opposite effects in the study cohort: increases in rifampicin Cmax and AUC were associated with reduced ALT values, while increases in AST were associated with elevated rifampicin levels. The linear regression models describing Cmax, AUCt and AUCi were strengthened when the AST/ALT ratio substituted ALT and AST (Tables 5, 10, 13). Kimerling et al.[74] found alcohol use to be associated with higher rifampicin levels, and chronic alcoholic liver disease is associated with increased AST/ALT ratios[144]. Reduced clearance of rifampicin due to alcohol-induced hepatic impairment may explain the findings in this study.

However, alcohol use (as assessed in this study) did not significantly affect the pharmacokinetics of rifampicin; this might reflect the weak tool used in this study to assess alcohol exposure. Alternatively, increased AST/ALT ratios may result directly from increased exposure to rifampicin. The fact that AST and ALT did not contribute significantly to the regression models when they were added as dichotomized variables (Table 6), suggests that subtle variability of the enzyme levels including that within the reference ranges is associated with rifampicin pharmacokinetics. The statistically significant correlations between total bilirubin levels and the AST/ALT ratio, and the measures of bioavailability are shown in figure 4.

Figure 4: The correlations between rifampicin Cmax (on the left axes: mg/l), AUC0- and AUC0 (both on the right axes: mg.hr/l) and (A) total bilirubin level, and (B) AST/ALT ratio. Evaluations of the statistical significance of the relationships were based on Spearman's rank correlations.



The inverse association of albumin levels and rifampicin bioavailability is surprising. Polasa et al.[64] found low rifampicin concentrations to be associated with low body mass index and low albumin levels; an effect that could be partly explained by drug distribution as rifampicin is highly protein bound, mostly to albumin[65]. Therefore, albumin should theoretically retain rifampicin in the systemic compartment. The argument is, however, jeopardized by the fact that the bonds between rifampicin to albumin are relatively weak[39]. In this study body mass index was not associated with rifampicin concentration. In contrast, haemoglobin levels were positively associated with rifampicin bioavailability, and patients with higher haemoglobin concentrations also absorbed the drug more efficiently, as evidenced by reduced Tmax values and decreased risk of a nonzero lag-time. Although albumin and haemoglobin would both tend to be reduced in debilitated

patients, they have opposite effects on the pharmacokinetics, perhaps haemoglobin and albumin are markers of specific disease effects or nutritional deficiencies with different pharmacokinetic consequences.

Although the ESR was inversely associated with Tmax, and rifampicin concentrations took longer to reach peak concentrations in previously treated patients, patients with increased total protein levels, urea concentrations, and white cell counts, these factors were not associated with altered Cmax or AUC values. Younger patients and patients with higher urea values were more likely to have nonzero lag times for rifampicin absorption.

Reduced elimination was associated with increasing age, creatinine concentrations, bilirubin levels, white cell counts and ESR. These findings are not surprising. Reduced renal drug elimination is known to be associated with increased age, and although rifampicin is largely eliminated by the liver, proportion is excreted in the urine. The competition of bilirubin and rifampicin for biliary excretion was mentioned previously. Increased white cell counts and ESR values, are likely to be associated with more severe illness and might also, therefore, be expected to be associated with reduced rates of drug elimination. Conversely, the positive association of red cell volume with rifampicin elimination rate might be attributed to more efficient drug excretion in patients with better nutrition, less protracted illness and less associated microcytic anaemia. Smoking was also associated with increased elimination rates. Smoking is associated with the induction of certain hepatic enzymes (eg. CYP2E1: and CYP1A2, which metabolizes theophylline and caffeine)[145], but its association with enhanced rifampicin elimination has not been described previously.

ISONIAZID

Two models were used to describe the effect of covariates on the Cmax, AUCt and AUCi of isoniazid. Acetylator status was determined on a sub-group of patients who were recruited largely before FDCs of rifampicin and isoniazid were introduced in the wards. Thus, only one subject with known acetylator status received a FDC product on the day of PK sampling. As both formulation type and acetylator status were important determinants of Cmax, AUCt and AUCi, it was necessary to use 2 regression models for each measure.

Regression of covariate factors on isoniazid Cmax

In the first model the effect of acetylator type was not evaluated. Two subjects with very low isoniazid concentrations (0.68 and 0.46 mg/l) were excluded from the data set and Cmax was log-transformed in order to satisfy the assumptions of the linear regression model. Twenty-eight percent of the variability in isoniazid peak concentrations was explained by this model (table 19). The type of formulation and sex were important determinants of isoniazid peak concentrations; males had peak concentrations 24.88% lower than females and the use of FDCs was associated with a 22.94% reduction. BMI and drug dose/kg displayed a degree of multi-colinearity, but as dose/kg strengthened the model more, BMI was not included. Each 1 mg/kg increase in the isoniazid dose/kg was associated with a 5.33% elevation of the peak concentration. Each unit increase in haemoglobin levels was associated with a 3.63% increase in the Cmax. Minor increases in the Cmax occurred with increased γ-GT and decreased total protein concentrations.

Table 19: Multiple linear regression model of covariate factors on the log-transformed isoniazid peak concentration (Cmax model A)

Source	SS	df	MS			Number of obs = 133
Model	4.9382	6	.8064			F(6, 126) = 8.12
Residual	12.5188	126	.0994			Prob > F = 0
Total	17.3569	132	.1315			R-squared = 0.2787
						Adj R-squared = 0.2444
Ln(Cmax)						Root MSE = 0.3152
	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	% change in Cmax for 1 unit increase
sex	-0.2861	0.0594	-4.82	0.000	-0.4036 -0.1687	↓24.88%
Drug dose/kg	0.0519	0.0229	2.27	0.025	0.0067 0.0971	↑5.33%
Formulation type	-0.2605	0.0689	-3.78	0.000	-0.3969 -0.1241	↓22.94%
Total protein	-0.0092	0.0038	-2.43	0.017	-0.0168 -0.0017	↓0.92%
γ-GT	0.0020	0.0007	2.69	0.008	0.0005 0.0034	↑0.20%
haemoglobin	0.0356	0.0178	2.00	0.048	0.0003 0.0709	↑3.63%
constant	1.9500	0.3826	5.10	0.000	1.1927 2.7072	

Amongst those patients with known acetylator type, the addition of acetylator status substantially strengthened the model describing the square root of Cmax, which described 40% of the variability associated with the dependent variable. However, the entry criteria for inclusion in the model (table 20) had to be broadened as the association was not significant at the 0.075 level. Intermediate and rapid acetylators tended to have lower peak isoniazid concentrations. The formulation type was not included in this model as all but one subject with known acetylator status received single drug products. The other covariates included with $p < 0.05$ were the same as those included in the previous model.

Table 20: Multiple linear regression model of covariate factors on the square root transformed isoniazid peak concentration: (Cmax model B)

Source	SS	df	MS	Number of obs = 91
Model	10.5309	8	1.3164	F(8, 82) = 6.97
Residue	15.4855	82	.1888	Prob > F = 0
Total	26.0163	90	.2891	R-squared = 0.4048
				Adj R-squared = 0.3467
Sqrt(Cmax)				Root MSE = 0.4346

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Acetylator type	-0.2126	0.1232	-1.73	0.088	-0.4577	0.0325
ESR	0.0032	0.0017	1.95	0.055	-0.0001	0.0065
Haemoglobin	0.0969	0.0345	2.81	0.006	0.0282	0.1656
Sex	-0.4022	0.1016	-3.96	0.000	-0.6043	-0.2001
Total protein	-0.0224	0.0071	-3.16	0.002	-0.0365	-0.0083
γ-GT	0.0032	0.0011	2.93	0.004	0.0010	0.0053
Platelet count	-0.0007	0.0004	-1.82	0.073	-0.0014	0.0001
Drug dose/kg	0.1217	0.0458	2.66	0.009	0.0306	0.2127
constant	2.8318	0.6321	4.48	0.000	1.5743	4.0892

Regression of covariate factors on isoniazid AUCt

In the first model (A: table 21), formulation type and the other covariates except for acetylator status were regressed. The covariate factors were regressed onto the square root of the area under the isoniazid concentration-time curve to 8 hours after drug dosing. The magnitude of the influence of covariate factors cannot be estimated when this transformation is used. However as for the peak concentration, male sex and FDC use were associated with significant AUCt decreases. As body mass index and isoniazid dose/kg (Spearman's rho: -0.6939, p = 0.0000), and γ-GT and alkaline phosphatase (Spearman's rho: 0.4048, p = 0.0000) displayed a degree of multi-colinearity, the variable in each pair that strengthened the model most was chosen for inclusion in the model. Increased AUCt values were associated with higher drug doses/kg, higher haemoglobin concentrations, higher urea levels (p = 0.072), higher γ-GT levels and increased erythrocyte sedimentation rates.

Table 21: Model A (Multiple linear regression model of covariate factors on the square root of AUCt. The effect of acetylator status was not assessed in this model.)

Source	SS	df	MS	Number of obs = 131
Model	40.7901	7	5.7557	F(7, 123) = 6.58
Residual	107.5905	123	.874/	Prob > F = 0.0000
Total	147.8806	130	1.1375	R-squared = 0.2725
				Adj R squared = 0.2310
Sqrt(AUCt)				Root MSE = .9353

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
γ-GT	0.0042	0.0020	2.12	0.036	0.0003	0.0082
sex	-0.5577	0.1851	-3.01	0.003	-0.9242	-0.1913
Drug dose/kg	0.1336	0.0721	1.85	0.066	-0.0091	0.2762
ESR	0.0068	0.0027	2.52	0.013	0.0015	0.0122
Haemoglobin	0.2087	0.0611	3.41	0.001	0.0876	0.3297
Urea	0.1426	0.0785	1.82	0.072	-0.0128	0.2980
Formulation type	-0.7546	0.2160	-3.49	0.001	-1.1821	-0.3270
constant	1.0206	1.0230	1.00	0.320	-1.0044	3.0456

In the second model (B; table 22), which accounted for 35 % of the variability of AUCt, formulation type was not used as a covariate. The 2 subjects with very low isoniazid concentrations were excluded and the square root of AUCt was used in order to satisfy the assumptions of the model. Multi-colinearity was observed between BMI and isoniazid dose/kg; BMI was dropped from the model as the contribution of drug dose/kg to the model was stronger. Females, slow acetylators, those receiving higher isoniazid doses per kilogram and those with higher γ-GT levels had greater AUCt values. Those with higher platelet counts had lower AUCt values. Creatinine showed positive correlations when 6 potentially influential observations were dropped, but was not important when the whole data set was used.

Table 22: Model B (Multiple linear regression model of covariate factors on the square root of AUCt. The effect of formulation type was not assessed in this model.)

Source	SS	df	MS	Number of obs = 88
Model	26.3126	5	5.2625	F(5, 82) = 8.99
Residual	47.9826	82	.5852	Prob > F = 0
Total	74.2952	87	.8540	R-squared = 0.3542
				Adj R squared = 0.3148
Sqrt(AUCt)				Root MSE = 0.7650

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
sex	-0.6646	0.1799	-3.69	0.000	-1.0226	-0.3067
Platelets	-0.0014	0.0006	-2.24	0.028	-0.0027	-0.0002
Drug dose/kg	0.2240	0.0818	2.74	0.008	0.0614	0.3867
Acetylator status	-0.6349	0.2120	-2.99	0.004	-1.0567	-0.2131
γ-GT	0.0036	0.0016	2.22	0.029	0.0004	0.0069
constant	4.9862	0.5225	9.54	0.000	3.9467	6.0257

In summary sex, formulation type, acetylator status and isoniazid dose/kg were important determinants of the AUCt. Increases in γ-GT were also consistently associated with higher AUCt values. While urea, haemoglobin, platelet count and ESR had minor contributions in one model or the other. The inconsistency of the associations of these variables between

the different models (with their differing data sets) suggests that the associations are weaker.

Regression of covariate factors on isoniazid AUCi

It was again necessary to create 2 regression models to describe the relationships between the covariate factors and AUCi, as both formulation type and acetylator status were influential.

In the first model (A; table 23) acetylator status was not entered as a factor. The dependent variable (AUCi) was transformed by taking the square root in order to satisfy the assumptions of the linear regression model. Mean cell volume and total bilirubin were significant in a model excluding potentially influential observations; but in the final model, including all observations, they were not significantly associated with AUCi. Males, patients with higher BMIs, and those who received FDCs had lower isoniazid AUCi values. Greater AUCi values were associated with higher urea levels and, interestingly, the presence of HIV-infection. The model explained only 21% of the variability associated with the square root of AUCi.

Table 23: Model A (Multiple linear regression model of covariate factors on the square root of AUCi. The effect of acetylator status was not assessed in this model)

Source	SS	df	MS		
Model	45.4694	7	6.4956	Number of obs = 137	
Residual	168.8030	129	1.3086	F(7, 129) = 4.96	
Total	214.2724	136	1.5755	Prob > F = 0.0001	
				R-squared = 0.2122	
				Adj R-squared = 0.1695	
Sqrt(AUCi)				Root MSE = 1.1439	

	Coef.	Std. Err.	T	P>|T|	[95% Conf.	Interval]
Sex	-0.5015	0.2088	-2.40	0.018	-0.9146	-0.0883
Urea	0.2104	0.0767	2.75	0.007	0.0588	0.3621
HIV-infection	0.7252	0.3511	2.07	0.041	0.0305	1.4199
Total bilirubin	0.0377	0.0258	1.46	0.146	-0.0133	0.0886
Mean cell volume	0.0005	0.0114	0.04	0.966	-0.0221	0.0231
BMI	-0.1295	0.0337	-3.84	0.000	-0.1961	-0.0628
Formulation type	-0.5247	0.2513	-2.09	0.039	-1.0219	-0.0274
Constant	7.2599	1.3129	5.53	0.000	4.6622	9.8575

Acetylator status was entered in the second model (B; table 24), and formulation status was omitted. The model explained 40% of the variability in AUCi amongst the 90 subjects with measurements of the relevant covariates. Intermediate and rapid acetylators, on average, had AUCi values 10.56 mg.hr.l^{-1} lower than slow acetylators. In keeping with the findings in A (above), HIV-infection was associated with an 8.10 mg.hr.l^{-1} increase in the

AUCi, and for each 1 mmol/l increase in urea the AUC was increased by 4.01 mg.hr.l^{-1}. Conversely, smoking and isoniazid dose/kg were determinants in model B: weak associations were found between γ-GT and platelet count and AUCi, and sex and BMI were not significant determinants. The differences between the models suggests that the associations with these variables are weaker, and may be due to biases in the distribution of the covariate factors in the different subject groups included in the models. Smoking was associated with a 5.8 mg.hr.l^{-1} increase in AUCi values, and for each 1mg/kg increase in isoniazid dose, the AUCi increased by 4.81 mg.hr.l^{-1}.

Table 24: Model B (Multiple linear regression model of covariate factors on AUCi. The effect of formulation type was not assessed in this model.)

Source	SS	DF	MS	Number of obs = 90
Model	6781.4975	7	968.7839	F(7, 82) = 7.96
Residual	9981.6981	82	121.7280	Prob > F = 0
Total	16763.186	89	188.3504	R-squared = 0.4045
				Adj R-squared = 0.3537
AUCi				Root MSE = 11.033

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
Smoking	5.8406	2.4795	2.36	0.021	0.9081	10.7731
Platelet count	-0.0168	0.0090	-1.86	0.067	-0.0348	0.0012
HIV-infection	8.0962	3.5872	2.26	0.027	0.9601	15.2324
Urea	4.0438	1.0884	3.72	0.000	1.8786	6.2090
Isoniazid dose/kg	4.8056	1.0971	4.38	0.000	2.6231	6.9882
Acetylator status	-10.5556	3.1019	-3.40	0.001	-16.7263	4.3850
γ-GT	0.0564	0.0249	2.26	0.026	0.0068	0.1059
Constant	-3.4992	8.5843	-0.41	0.685	-20.5761	13.5776

Regression of covariate factors on isoniazid elimination rate constant (ke) and half-life

Formulation type was not related to isoniazid elimination. The model derived (Table 25) accounts for 27% of the variability in the elimination rate constant of isoniazid amongst the 92 subjects with data for all the covariates included in the model. Intermediate and rapid acetylators had ke values 23% greater than slow acetylators. The elimination rate constant was reduced by 25% in HIV-infected patients compared to those without HIV-infection. For each unit increase in haemoglobin or urea, there were 8% increments and 5% reductions in the ke, respectively. Small increases in elimination were associated with ALT, MCV and ESR; and small decreases were seen with increased levels of total protein.

The relationship of half-life to the covariate factors is shown in Table 26. The relationships reflect those seen for elimination rate constant.

Table 25: Multiple linear regression model describing covariate factors on the log transformed elimination rate constant.

Source	SS	df	MS	Number of obs = 92
Model	3.8273	8	0.4784	F(8, 83) = 3.79
Residual	10.4722	83	0.1262	Prob > F = 0.0008
Total	14.2994	91	0.1571	R-squared = 0.2677
				Adj R-squared = 0.1971
Ln(ke)				Root MSE = 0.35521

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]		% change in ke for 1 unit increase
HIV-infection	-0.2835	0.1229	2.31	0.024	-0.5279	-0.0391	↓24.68%
Total protein	-0.0154	0.0054	-2.85	0.005	-0.0261	-0.0046	↓1.52%
Haemoglobin	0.0724	0.0279	2.59	0.011	0.0168	0.1279	↑7.50%
ALT	0.0081	0.0044	1.86	0.066	-0.0006	0.0168	↑0.81%
Urea	-0.0544	0.0262	-2.08	0.041	-0.1065	-0.0023	↓5.29%
MCV	0.0080	0.0042	1.90	0.061	-0.0004	0.0164	↑0.80%
ESR	0.0032	0.0013	2.45	0.016	0.0006	0.0058	↑0.32%
Acetylator status	0.2067	0.0995	2.08	0.041	0.0087	0.4046	↑22.96%
Constant	-1.9301	0.6207	-3.11	0.003	-3.1647	-0.6955	

Table 26: Multiple linear regression model describing covariate factors on the log-transformed half-life of isoniazid.

Source	SS	df	MS	Number of obs = 92
Model	3.8274	8	0.4784	F(8, 83) = 3.79
Residual	10.4727	83	0.1262	Prob > F = 0.0008
Total	14.3001	91	0.1571	R-squared = 0.2676
				Adj R squared = 0.1971
Ln(half-life)				Root MSE = 0.3552

	Coef.	Std. Err.	t	P>|t|	[95% Conf. interval]		% change in ke for 1 unit increase
HIV-infection	0.2834	0.1229	2.31	0.024	0.0390	0.5279	↑32.77%
Total protein	0.0154	0.0054	2.85	0.005	0.0046	0.0261	↑1.55%
Haemoglobin	-0.0724	0.0279	-2.59	0.011	-0.1279	-0.0168	↓6.98%
ALT	-0.0081	0.0044	-1.86	0.066	-0.0168	0.0006	↓0.81%
Urea	0.0544	0.0262	2.08	0.041	0.0023	0.1065	↑5.59%
MCV	-0.0080	0.0042	-1.90	0.061	-0.0164	0.0004	↓0.80%
ESR	-0.0032	0.0013	-2.45	0.016	-0.0058	-0.0006	↓0.32%
Acetylator Status	-0.2066	0.0995	2.08	0.041	-0.4046	-0.0087	↓18.67%
constant	1.5636	0.6207	2.52	0.014	0.3290	2.7981	

Regression of covariate factors on delayed peak levels (Tmax ≥ 3 hours) of isoniazid

The assumption of the linear regression models using Tmax, ln(Tmax) and sqrt(Cmax) as a continuous variable could not be satisfied. Tmax was therefore dichotomized into values below 3 hours (variable=0) and ≥ 3hours (variable=1) as patients with a Tmax of 3 hours or more comprised a minority of 13% with considerably delayed peak concentrations in comparison to most patients. Logistic regression was used to determine the associations between the covariate factors and delayed peak concentrations (table 27). FDC use was associated with a 9.89 times greater risk of a late Tmax (≥ 3 hours) than the use of tablets containing isoniazid alone, and males were 4.25 times more likely to have

delayed peak drug levels. Alcohol use in the year prior to admission was weakly associated with a 72% reduction in the risk of delayed peak concentrations. For each g/dl increase in haemoglobin, the risk of delayed Cmax was reduced by 43%. Each unit increase in AST, albumin and white cell count, was associated with an 8%, 16% and 19% increased risk, respectively, of a late Tmax. No HIV-infected individuals had a Tmax ≥ 3 hours.

Table 27: Logistic regression model describing the relative risk associated with covariates of peak Isoniazid concentrations occurring at or later than 3 hours.

Logistic estimates					Number of obs = 137
LR chi2(7) = 27.07					Pseudo R2 = 0.2540
					Prob > chi2 = 0.0003
Tmax>3hours					Log likelihood = -39.7602
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]
sex	4.2525	2.9194	2.11	0.035	1.1074 16.3305
alcohol use	0.2838	0.1879	-1.90	0.057	0.0775 1.0393
AST	1.0750	0.0366	2.12	0.034	1.0055 1.1493
Albumin	1.1577	0.0885	1.92	0.055	0.9967 1.3448
haemoglobin	0.5737	0.1259	-2.53	0.011	0.3731 0.8820
White cell count	1.1908	0.0954	2.15	0.029	1.0177 1.3933
formulation type	9.8929	6.9277	3.27	0.001	2.5076 39.0096

Discussion of the determinants of isoniazid PK measures

The patient and treatment factor predictors of the PK measures for isoniazid are summarized in table 28.

Table 28: Summary of the covariate factors influencing Isoniazid pharmacokinetic measures, as determined by regression analyses. Only those variables contributing to the final models are included. Multiple linear regression was used for all dependent variables except Tmax, for which logistic regression analysis was used after dichotomizing the variable (those with a Tmax of ≥ 3 hours = 1, and those with Tmax < 3 hours after drug administration = 0).

	Isoniazid								
PK measure	Cmax		AUC		AUCi		Ke	t1/2	Tmax
	A	B	A	B	A	B			
Transformation	ln	sqrt	sqrt	sqrt	sqrt		ln	ln	0<3hr / ≥3h
R-squared for model	0.28	0.40	0.27	0.38	0.21	0.40	0.27	0.27	0.25 log-lik
Covariate factors:									
Age (years)	↔	↔	↔	↔	↔	↔	↔	↔	↔
Sex (female=0; male=1)	↓	↓	↓	↓	↓	↔	↔	↔	↑
Smoking? (no=0; yes=1)	↔	↔	↔	↔	↔	↑	↔	↔	↔
Alcohol? (no=0; yes=1)	↔	↔	↔	↔	↔	↔	↔	↔	↓
Treatment category? ('new'=0; retreatment'=1)	↔	↔	↔	↔	↔	↔	↔	↔	↔
Formulation type? (SD=0; FDC=1)	↓		↓		↓		↔	↔	↑

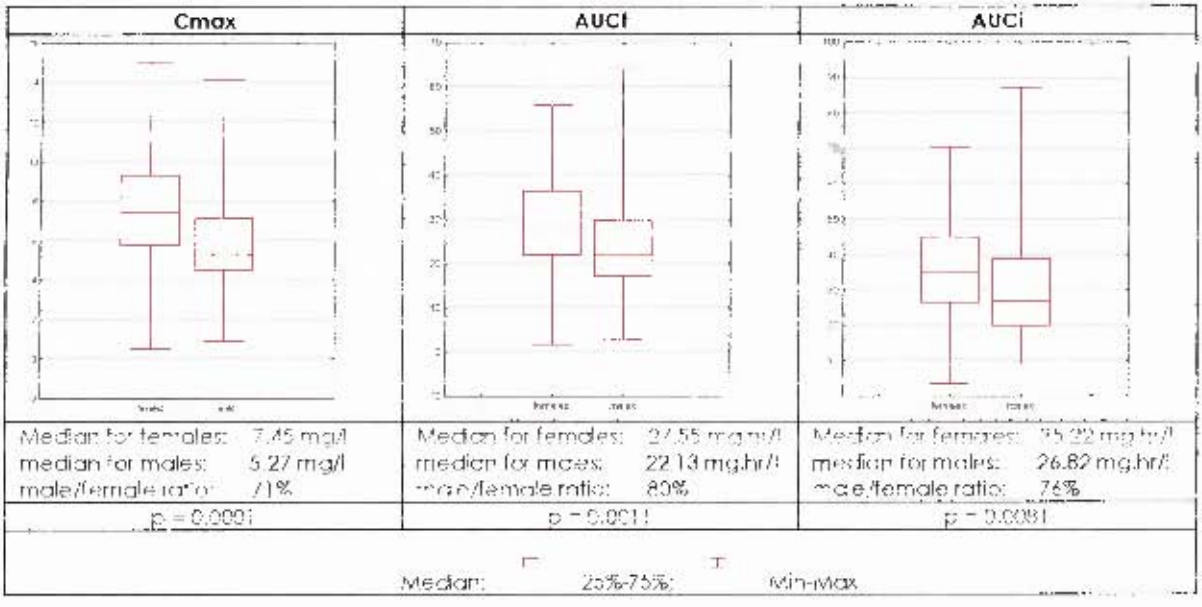
Table 28 continued

Isoniazid dose/kg	↑	↑	↑ (0.066)	↑	↔	↑	↔	↔	↔
BMI (kg/m²)	↔	↔	↔	↔	↓	↔	↔	↔	↔
Acetylator status (slow=0; intermediate/rapid=1)		↓		↓		↓	↑	↓	↔
Total protein (g/l)	↓	↓	↔	↔	↔	↔	↓	↑	↔
Albumin (g/l)	↔	↔	↔	↔	↔	↔	↔	↔	↑ (0.055)
Total bilirubin (µmol/l)	↔	↔	↔	↔	↔	↔	↔	↔	↔
AST (units/l)	↔	↔	↔	↔	↔	↔	↔	↔	↑
ALT (units/l)	↔	↔	↔	↔	↔	↔	↑ (0.066)	↓ (0.066)	↔
AP (units/l)	↔	↔	↔	↔	↔	↔	↔	↔	↔
γ-GT (units/l)	↑	↑	↑	↑	↔	↑	↔	↔	↔
Urea (mmol/l)	↔	↔	↑ (0.072)	↔	↑	↑	↓	↑	↔
Creatinine (µmol/l)	↔	↔	↔	↔	↔	↔	↔	↔	↔
Haemoglobin (g/dl)	↑	↑	↑	↔	↔	↔	↑	↓	↓
MCV (fl)	↔	↔	↔	↔	↔	↔	↑ (0.061)	↓ (0.061)	↔
WCC (10⁹/l)	↔	↔	↔	↔	↔	↔	↔	↔	↑
Platelets (10⁹/l)	↔	↓	↔	↓	↔	↓	↔	↔	↔
ESR (mm)	↔	↑ (0.055)	↑	↔	↔	↔	↑	↓	↔
HIV antibodies (Elisa negative=0; positive=1)	↔	↔	↔	↔	↑	↑	↓	↑	↔
CD4/cells/ml)	↔	↔	↔	↔	↔	↔	↔	↔	↔

↑: the variable contributed significantly to the model describing the PK measure; an increase in the relevant variable was associated with an increase in the value of the PK measure. P-values > 0.05 are specified.
↓: the variable contributed significantly to the model describing the PK measure; a decrease in the relevant variable was associated with a smaller value for the PK measure. P-values > 0.05 are specified.
↔: no significant contribution to the regression model describing the PK measure
0: no observations with tmax ≥ 3 hours
[1] history of smoking in the year prior to admission to Brewelskloof Hospital
[2] history of regular alcohol consumption in the year prior to admission to Brewelskloof Hospital
[3] patients in 'new' category had not received more than 1 month of drug treatment prior to admission; patients in the 'retreatment' category had previously received antituberculosis drugs for one month or longer.
[4] SD: Single drug formulations were used; Tablets or capsules each containing a single drug were administered together.
FDC: Rifampicin-containing fixed dose combination formulations were used. Rifampicin was combined with isoniazid in RiFinah® (rifampicin 300 mg with isoniazid 150 mg, or rifampicin 150 mg with isoniazid 75 mg); single drug formulations of pyrazinamide and ethambutol were used.

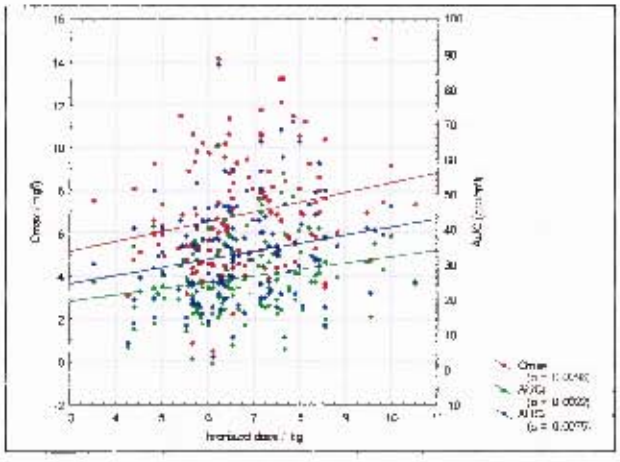
Isoniazid had substantially superior bioavailability in females, and peak concentrations were delayed in males. This effect was independent of the dose/kg and sex was not a significant determinant of the rate of elimination. An explanation for the finding is not apparent; differences in drug distribution between the males and females may be important, although isoniazid is readily distributed to most tissues[44]. The statistically significant effect of sex, without adjustment for other covariates, is illustrated in figure 5.

Figure 5: Box and whisker plots of isoniazid Cmax, AUC1 and AUCi, by sex. The statistical significance of the differences between males and females for each PK measure were evaluated using the Kruskal Wallis test.



The dose of isoniazid per kilogram of body weight was also an important determinant of systemic isoniazid concentrations (figure 6). Drug dose/kg and body mass index displayed a degree of multi-colinearity, but the association of dose/kg with bioavailability was stronger in most instances.

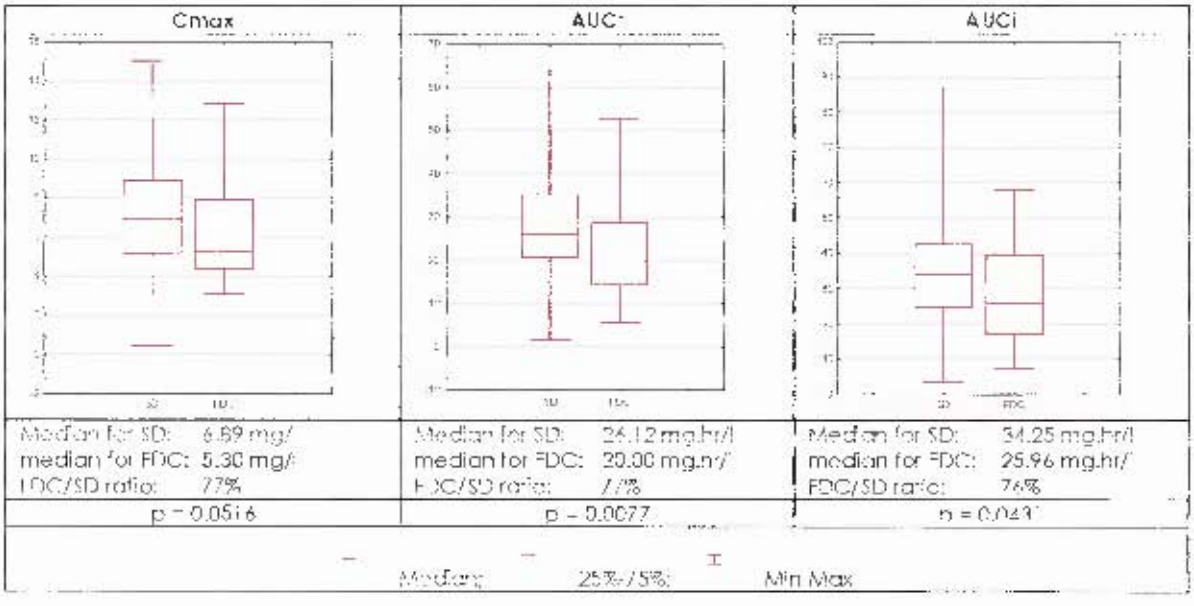
Figure 6: The relationship of isoniazid Cmax, AUC1 and AUCi with the dose/kg. The statistical significance of the associations was determined using Spearman's rank correlations.



The use of FDCs was associated with reduced bioavailability and delayed peak concentrations of isoniazid, as was seen for rifampicin. The bioavailability of isoniazid in

FDCs is not generally perceived to be problematic. However, the 'on-the-shelf' interaction alluded to earlier, between rifampicin and isoniazid occurring with exposure to heat, humidity and light[97] did lead to small reductions in the isoniazid content of the products studied. In this study the differences were significant without adjustment for other covariates), and the reductions were substantial (figure 7).

Figure 7: The differences in Cmax, AUC1 and AUC2 between those who received formulations containing isoniazid alone (SDs) and those who received FDCs of isoniazid and rifampicin. The significance of the differences between the SD and FDC groups was evaluated using the Kruskal-Wallis test.



As might be expected, acetylator type was clearly an important determinant of elimination rate and bioavailability. Although the multivariate regressions showed a weak association with Cmax, reductions in AUC1 and half-life of approximately 10.56 mg.hr.l^{-1} and 21%, respectively, were observed amongst intermediate and rapid acetylators when compared to slow acetylators (tables 24 and 26). The differences in Cmax, AUC1 and AUC2 between slow, and intermediate and fast acetylators (without adjustment for other covariates) is shown in figure 8.

Reduced elimination rates and higher AUC1 values were associated with HIV-infection, although without adjustment for the other covariates, the differences were not statistically significant (figure 9). The trends are consistent with the findings of O-Neil et al.[63], who described conversion from fast to slow acetylator phenotype with the progression of HIV infection. Although the differences in the Cmax and AUC between the groups in Sahai's study[65] were not significant, asymptomatic HIV-infected individuals

without diarrhea tended to have slightly higher mean Cmax and AUC values than the healthy controls, while those with CD4+ cell counts below 200 and diarrhea had lower mean Cmax and AUC values than the control group. The half-lives of isoniazid were similar between the groups in Sahai's study.

Figure 8: The differences in Cmax, AUCt and AUCi by acetylator type. The significance of the differences between the groups was evaluated using the Kruskal-Wallis test.

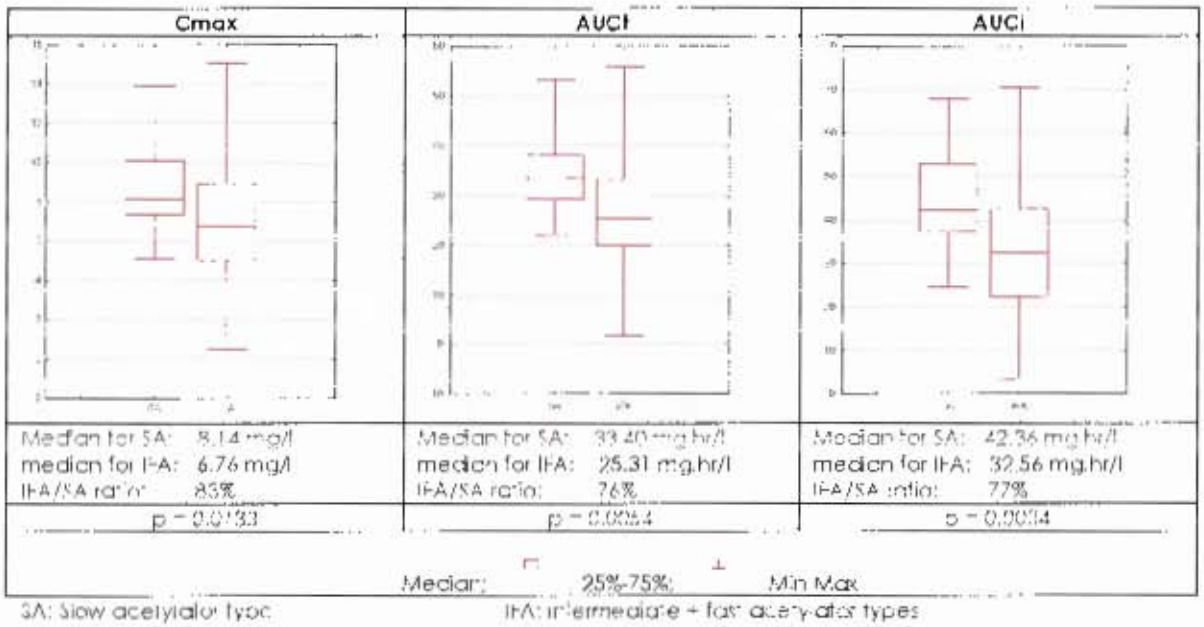
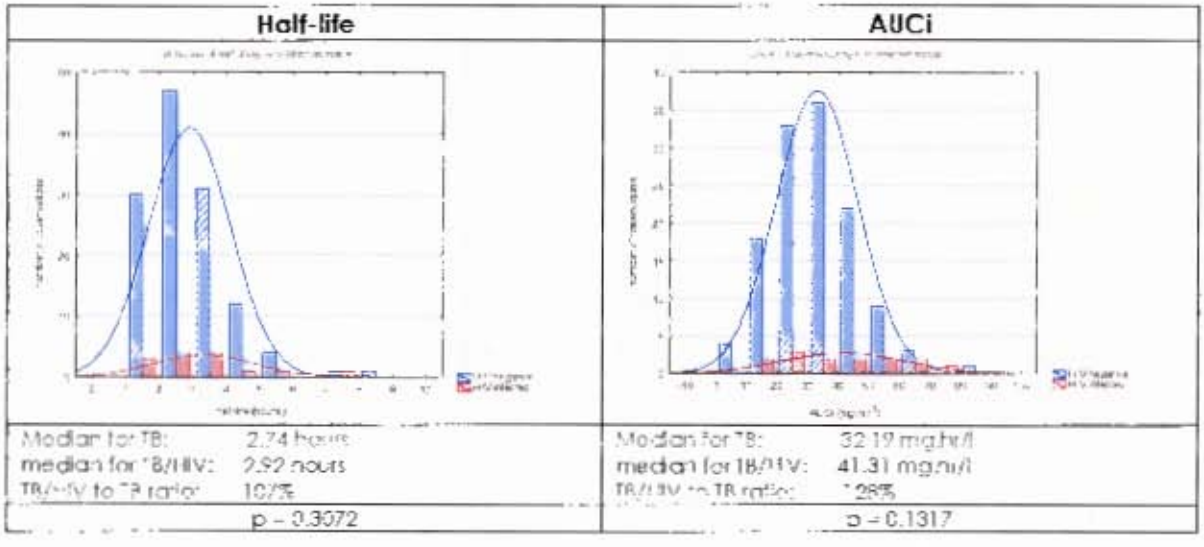


Figure 9: The effect of HIV infection on the distribution of isoniazid half-life and AUCi, respectively.



Increased levels of urea were also associated with reduced elimination rates and increased AUC values; findings consistent with the renal elimination of isoniazid, albeit

mostly as metabolites. Although the correlation between urea and the elimination rate constant was statistically significant without adjustment for the other covariates, the effect was not substantial (Spearman's rho: -0.1793, p = 0.0336), in this group of patients with largely adequate renal function.

The increased levels of isoniazid seen in patients with higher γ-GT levels could be related to reduced elimination, although γ-GT was not included as a significant determinant in the models describing the elimination rate constant and half-life.

Delayed peak concentrations were also associated with increased white cell counts and AST levels; associations which might be related to delayed absorption in more severely ill patients. Admission to alcohol consumption in the year prior to admission and lower albumin levels were weakly associated with a reduced risk of delayed absorption. These factors were not associated with altered bioavailability.

Those with increased platelet counts and total protein tended to have reduced isoniazid bioavailability, and total protein was also associated with reduced elimination; effects possibly associated with more advanced disease. Conversely, higher erythrocyte sedimentation rates were associated with marginally more efficient elimination, and a tendency to higher isoniazid levels. Haemoglobin was more strongly associated with earlier and higher peak concentrations, in spite of more efficient elimination. Increased MCV was also associated with more efficient elimination, possibly indicating reduced elimination in those with more marked reductions in iron utilization or supply. ALT was weakly associated with more efficient elimination.

Those with a history of smoking had increased AUCi values. Aromatic amines from cigarette smoking are metabolized by N-acetyltransferases, thus competitive inhibition of isoniazid by these substances in smokers might be involved. Conversely, smoking induces CYP2E1[145], which is involved in the metabolism of isoniazid, but this is a minor metabolic pathway.

PYRAZINAMIDE

Regression of covariate factors on pyrazinamide peak concentrations

To satisfy the assumptions of the linear regression model, one extreme outlying observation (subject 87, who had very low drug concentrations; see chapter III) was dropped from the data set. The model (table 29) explained 47% of the variability associated with the peak levels of pyrazinamide. Smoking in the year prior to admission and male sex were associated with 5.23 and 4.70 mg/l reductions in pyrazinamide Cmax, respectively. For each 1 mg/kg increase in the dose of pyrazinamide the Cmax was increased by 1.31 mg/l. Each unit increase in total bilirubin was associated with a 0.41 mg/l increase in the Cmax.

Table 29. Multiple linear regression model describing the effect of patient and treatment factors on the peak concentration of pyrazinamide.

Source	SS	df	MS	Number of obs = 137
Model	9682.2586	4	2420.5647	F(4, 132)=28.71
Residual	11129.713	132	84.3160	Prob > F =0
Total	20811.971	136	153.0292	R-squared =0.4652
				Adj R-squared =0.449
Cmax				Root MSE =9.1824

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
Sex	-4.7014	1.5913	2.95	0.004	-7.8493	-1.5536
Smoking	5.2282	1.7271	-3.03	0.003	-8.6445	-1.8119
Total bilirubin	0.4069	0.2023	2.01	0.046	0.0067	0.8072
Drug dose/kg	1.3102	0.1347	9.72	0.000	1.0437	1.5767
constant	10.9042	5.1380	2.12	0.036	0.7408	21.0676

Regression of covariate factors on pyrazinamide AUCt

Subject 87 was again dropped in order to satisfy the model assumptions. The model accounted for 46% of the variation in AUCt (table 30). As for Cmax, increases in drug dose/kg and total bilirubin were associated with increased AUCt values. Similarly, although the association was weaker (p=0.063), smokers had AUCt reductions of 20.57 mg.hr.l^{-1}. ALT and AST contributed significantly to a model without 8 potentially influential observations. The transaminases were therefore included in the final model although their inclusion reduced the significance of the association of AUCt with age; a degree of covariance between age and the transaminases within the model explains this phenomenon.

Table 30: Multiple linear regression model describing the effect of patient and treatment factors on the AUCi of pyrazinamide.

Source	SS	Df	MS	
Model	333502.11	6	55583.684	Number of obs = 135
Residual	397329.44	128	3104.1362	F(6, 128) = 17.91
Total	730831.54	134	5453.9668	Prob > F = 0
				R-squared = 0.4563
				Adj R-squared = 0.4308
AUCi				Root MSE = 55.715

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Total bilirubin	3.2061	1.2374	2.59	0.011	0.7578	5.6545
Age	0.6428	0.4194	1.53	0.128	-0.1872	1.4727
Smoking	-20.5735	10.9834	-1.87	0.063	-42.3059	1.1590
ALT	-1.1380	0.6194	-1.84	0.069	-2.3636	0.0877
AST	1.1715	0.7816	1.50	0.136	-0.3750	2.7180
Drug dose/kg	7.3278	0.8290	8.84	0.000	5.6874	8.9682
constant	3.7562	37.2118	0.10	0.920	-69.8736	77.3861

Regression of covariate factors on pyrazinamide AUCi

Three observations with the most leverage were dropped in order to satisfy the assumptions of the linear regression model (subject 17 had high bilirubin levels; 93 had a very high AUCi and high urea levels; and 153 had a low AUCi value). Forty-two percent of the variation in AUCi was accounted for by the model (table 31). HIV-infection, smoking and higher urea levels were associated with large reductions in the AUCi (108.43 mg.hr.l^{-1} for HIV-infection, 96.20 mg.hr.l^{-1} for smoking and 50.86 mg.hr.l^{-1} for each mmol/l of urea). Each 1 umol/l increase in total bilirubin and each 1 mg/kg increase in the dose of pyrazinamide were associated with increases of 17.50 and 12.12 mg.hr.l^{-1} increases in the AUCi. Increases in age and total protein were associated with smaller increments in AUCi.

Table 31: Multiple linear regression model describing the effect of patient and treatment factors on the AUCi of pyrazinamide.

Source	SS	Df	MS	
Model	2115853	7	302264.71	Number of obs = 132
Residual	2978291.3	124	24018.478	F(7, 124) = 12.58
Total	5094144.2	131	38886.597	Prob > F = 0
				R-squared = 0.4154
				Adj R-squared = 0.3823
AUCi				Root MSE = 154.98

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
HIV infection	-108.4334	50.9605	-2.13	0.035	-209.2987	-7.5685
Age	3.6630	1.2132	3.02	0.003	1.2617	6.0644
Smoking	-96.1995	30.7655	3.13	0.002	157.0930	-35.3059
Total protein	3.7186	1.7974	2.07	0.041	0.1611	7.2762
Bilirubin	17.4958	4.2174	4.15	0.000	9.1484	25.8432
Urea	-50.8563	15.2941	-3.33	0.001	-81.1276	-20.5851
Pyrazinamide dose/kg	12.1051	2.4731	4.89	0.000	7.2102	17.0000
constant	-181.8783	178.3336	-1.02	0.310	534.8505	171.0938

Regression of covariate factors on pyrazinamide elimination rate constant (ke) and half-life

The square root was used to satisfy the model assumptions. The magnitudes of the covariate influences on the untransformed values are thus not readily available from the model. However, statements about the direction of any associations can be made. The model accounted for 21% of the variability observed in sqrt(ke), (table 32). Males and patients with higher total bilirubin levels had reduced elimination rate constants, while increased ALT and urea values were associated with increased ke values.

Table 32: Multiple linear regression model describing the effect of patient and treatment factors on the square root of the elimination rate constant for pyrazinamide.

Source	SS	df	MS	Number of obs = 141
Model	0.0757	4	0.0189	F(4, 136) = 9.05
Residual	0.2842	136	0.0021	Prob > F = 0
Total	0.3599	140	0.0026	R-squared = 0.2102
				Adj R-squared = 0.187
sqrt(ke)				Root MSE = 0.0457

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	interval]
sex	-0.0230	0.0081	-2.86	0.005	-0.0390	-0.0071
Total bilirubin	-0.0031	0.0010	-3.14	0.002	-0.0051	-0.0012
ALT	0.0008	0.0004	2.17	0.032	0.0001	0.0016
Urea	0.0074	0.0030	2.46	0.015	0.0015	0.0134
constant	0.3371	0.0162	20.75	0.000	0.3049	0.3692

Table 33: Multiple linear regression model describing the effect of patient and treatment factors on the log-transformed half-life of pyrazinamide.

Source	SS	df	MS	Number of obs = 141
Model	2.6073	4	0.6518	F(4, 136) = 9.29
Residual	9.5429	136	0.0702	Prob > F = 0
Total	12.1502	140	0.08679	R-squared = 0.2146
				Adj R-squared = 0.1915
Ln (half-life)				Root MSE = 0.2649

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]	% change in AUC0-t for each 1 unit increase
sex	0.1381	0.0467	2.95	0.004	0.0457	0.2305	1.1481
Total bilirubin	0.0194	0.0055	3.56	0.001	0.0086	0.0302	1.0196
ALT	-0.0045	0.0022	-2.09	0.039	-0.0088	-0.0002	0.0045
Urea	-0.0399	0.0175	-2.28	0.024	-0.0745	-0.0054	0.0391
constant	1.8049	0.0941	19.17	0.000	1.6188	1.9911	6.0795

The natural logarithms of the dependent variable were used to satisfy the assumptions of the model describing the half life (table 33). As expected, in comparison to those for ke above, the inverse relationships were reflected. The half-life was 15% longer in males than females and for each unit increase in the total bilirubin, a 2% increase in the half-life was

observed. Single unit increases in urea and ALT were associated with reductions of 4% and less than 0.5%, respectively, in the half life.

Regression of covariate factors on the time to reach peak pyrazinamide

Pyrazinamide Tmax was largely independent of the covariate factors recorded. Delayed peak concentrations occurring 3 hours or more after drug ingestion in 18 patients, were not associated with any of the risk factors in a logistic regression model. A multiple linear regression model (with square root transformation of Tmax) explained only 5% of the variability observed, and showed that platelet count and white cell count had small but statistically significant associations (table 34).

Table 34: Multiple linear regression model describing the effect of patient and treatment factors on the time to reach peak concentrations of pyrazinamide.

Source	SS	df	MS	Number of obs = 142
Model	0.6942	2	.3471	F(2, 139) = 3.73
Residual	12.9523	139	.0932	Prob > F = 0.0266
Total	13.6466	141	.0968	R-squared = 0.0509
				Adj R-squared = 0.0372
Sqrt (Tmax)				Root MSE = 0.3053

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
White cell count	0.0209	0.0083	2.43	0.016	0.0038	0.0367
Platelet count	-0.0004	0.0002	-2.28	0.024	-0.0008	0.0001
constant	1.3172	0.0523	16.00	0.000	1.1544	1.4800

Discussion of covariate factor determinants of pyrazinamide PK measures

The patient and treatment covariate factor effects on the PK measures of pyrazinamide are summarized in table 35. Less variability of the PK measures was observed for pyrazinamide, compared to rifampicin, isoniazid and ethambutol, and the models describing the Cmax and AUC values explained a greater proportion of the associated variability. The variability of Tmax was poorly accounted for by the covariates recorded.

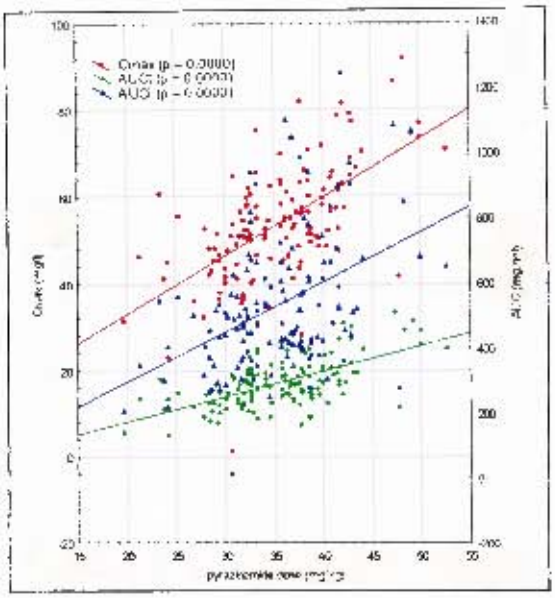
The drug dose per kilogram of body weight (ranging from 19.69 to 52.63 mg/kg) was the most important determinant of pyrazinamide concentrations. The Cmax, AUCt and AUCi increased in a linear manner with the weight adjusted dose (figure 10).

Table: 35: Summary of the covariate factors to influencing pyrazinamide pharmacokinetic measures as determined by regression analyses. Only those variables contributing to the final models are included. Multiple linear regression was used for all dependent variables except Tmax, for which logistic regression analysis was used after dichotomizing the variable (those with a Tmax ≥ 3 hours = 1, and those with a Tmax < 3 hrs = 0).

Pyrazinamide						
PK measure	Cmax	AUC$^{\infty}$	AUCt	Ke	T½	Tmax
Transformation	-	-	-	*sqrt*	*ln*	*sqrt*
R-squared for model	0.47	0.46	0.42	0.21	0.27	0.05
Covariate factors:						
Age (years)	↔	↔	↑	↔	↔	↔
Sex (female=0; male=1)	↓	↔	↔	↓	↑	↔
Smoking[1] (no=0; yes=1)	↓	↓	↓	↔	↔	↔
Alcohol[2] (no=0; yes=1)	↔	↔	↔	↔	↔	↔
Treatment category[3] ('new'=0; 'retreatment'=1)	↔	↔	↔	↔	↔	↔
Pyrazinamide dose/kg	↑	↑	↑	↔	↔	↔
BMI (kg/m²)	↔	↔	↔	↔	↔	↔
Total protein (g/l)	↔	↔	↑	↔	↔	↔
Albumin (g/l)	↔	↔	↔	↔	↔	↔
Total bilirubin (µmol/l)	↑	↑	↑	↓	↑	↔
AST (units/l)	↔	↔	↔	↔	↔	↔
ALT (units/l)	↔	↓	↔	↑	↓	↔
AP (units/l)	↔	↔	↔	↔	↔	↔
γ-GT (units/l)	↔	↔	↔	↔	↔	↔
Urea (mmol/l)	↔	↔	↓	↑	↓	↔
Creatinine (µmol/l)	↔	↔	↔	↔	↔	↔
Haemoglobin (g/dl)	↔	↔	↔	↔	↔	↔
MCV (fl)	↔	↔	↔	↔	↔	↔
WCC (10⁹/l)	↔	↔	↔	↔	↔	↑
Platelets (10⁹/l)	↔	↔	↔	↔	↔	↓
ESR (mm)	↔	↔	↔	↔	↔	↔
HIV antibodies (Elisa negative=0; positive=1)	↔	↔	↓	↔	↔	↔
CD4(cells/ml)	↔	↔	↔	↔	↔	↔

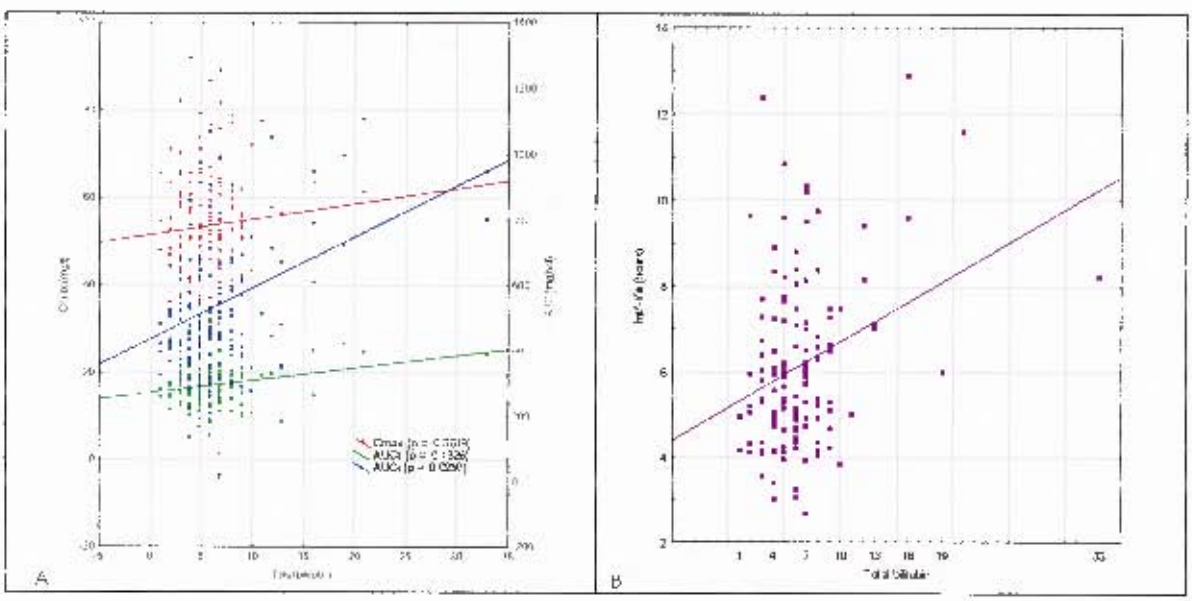
↑: the variable contributed significantly to the model describing the PK measure; an increase in the relevant variable was associated with an increase in the value of the PK measure. P-values > 0.05 are specified.
↓: the variable contributed significantly to the model describing the PK measure; a decrease in the relevant variable was associated with a smaller value for the PK measure. P-values > 0.05 are specified.
↔ no significant contribution to the regression model describing the PK measure
[1] history of smoking in the year prior to admission to Brewelskloof Hospital
[2] history of regular alcohol consumption in the year prior to admission to Brewelskloof Hospital
[3] patients in 'new' category had not received more than 1 month of drug treatment prior to admission; patients in the 'retreatment' category had previously received antituberculosis drugs for one month or longer.

Figure 10: Pyrazinamide Cmax, AUC1 and AUC1 vs. pyrazinamide dose/kg. The significance of the associations was tested using Spearman's rank correlations. (Cmax: rho= 0.5894, p=0.0000; AUC1: rho=0.5573, p=0.0000; AUC1: rho=0.4058, p=0.0000).



Total bilirubin concentration was positively associated with the half-life (spearman's rho: 0.2228; p = 0.0079) and the corresponding increases in pyrazinamide levels, reflected by the Cmax, AUC1 and AUC1 (figure 11). Reduced hepatic function (or possibly competition for hepatic elimination) may account for the association, with reduced elimination rates of both bilirubin and pyrazinamide.

Figure 11: The association between total bilirubin and (A) pyrazinamide Cmax, AUC1 and AUC1 (the statistical significance of the associations, without adjustment for other covariates, was established using Spearman's rank correlations), and (B) the half-life of pyrazinamide (Spearman's rho: 0.2228; p = 0.0079).

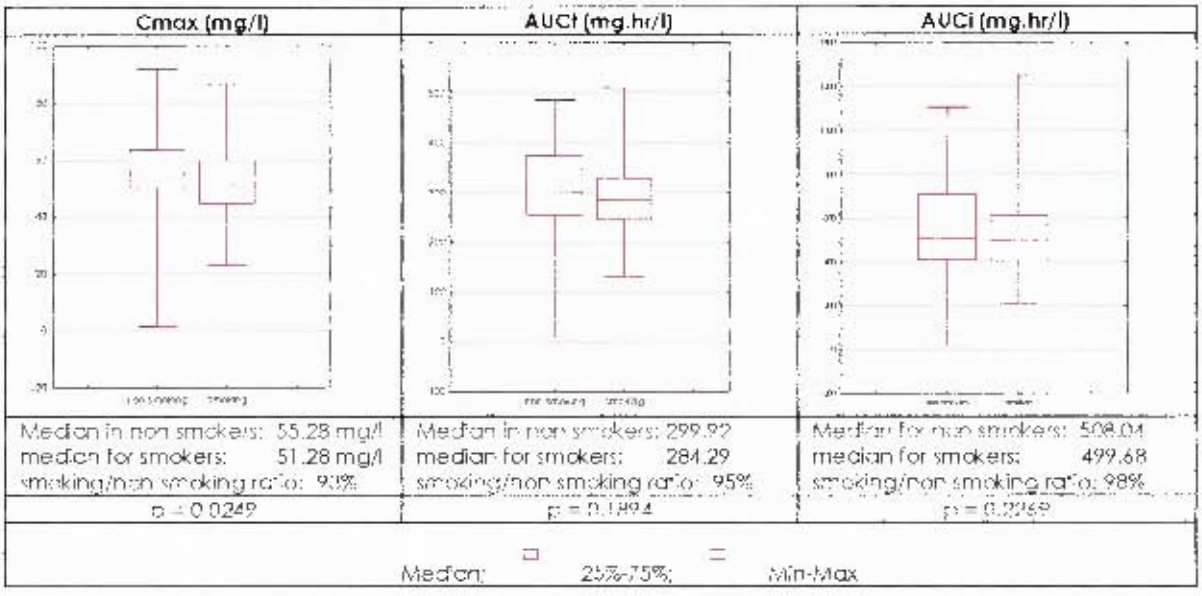


While men had reduced peak concentrations, they also eliminated the drug more slowly. Advanced age and higher total protein levels and HIV-negative status were associated with increased AUCi values, but were not important determinants of the elimination rate.

A reduction of approximately 24% in the pyrazinamide AUC was described by Sahai et al.[69] in HIV-infected individuals in comparison to healthy controls. Because square root transformation of the AUCi was used (in this study) for the regression model, it was not possible to estimate the reduction due to HIV infection adjusted for the other factors. The median AUCi in co-infected patients was approximately 12% lower than that in patients with tuberculosis alone, and the effect of HIV infection was not statistically significant without adjustment for the other covariates. The more dramatic reductions shown by Sahai et al. might be attributed to the healthy control group and a greater proportion of HIV infected subjects with more advanced disease.

Smoking was associated with reduced pyrazinamide levels, but was not a statistically significant determinant of the elimination rate. Without adjustment for other covariates, the reduction in the levels was small, but the effect was significant for Cmax (figure 12).

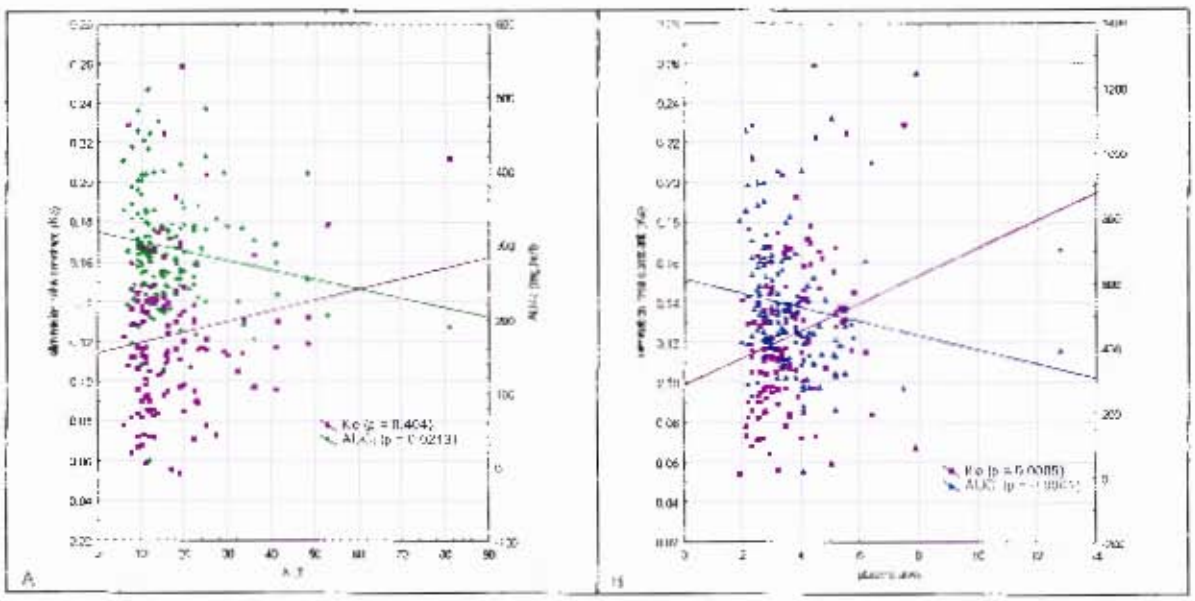
Figure 12: The effect of smoking on pyrazinamide Cmax, AUCt and AUCi. The significance of the effects was evaluated using the Kruskal Wallis test[4].



Increased AST and urea levels were associated with increased elimination rates and reductions in AUCt and AUCi, respectively. The correlations without adjustment for other

covariates are shown in figure 13: the AUCs were significantly reduced with increasing ALT and urea levels, while the association with elimination rate was significant only for ALT. Increased elimination in those with higher ALT levels is possibly explained by the association of relatively lower ALT levels with impaired hepatic function related to alcohol exposure, although this hypothesis is not supported by an association of elimination rate with a history of alcohol use in the year prior to admission. The relationship between urea levels and pyrazinamide is in contrast to that observed for isoniazid: higher urea levels were associated with increased elimination rates and reduced AUCI values. The effect might be explained if increased urea is associated with more efficient elimination related to better health and nutritional status in this patient group (indeed, there was a positive association between urea and BMI; table 2a).

Figure 13: The association of (A) ALT levels with the elimination rate and AUCI; and (B) urea levels with elimination rate and AUCI. The significance of the associations was tested using Spearman's rank correlations.



Increased white cell and decreased platelet counts were associated with delayed times to Cmax and accounted for a small amount of the variation in tmax.

ETHAMBUTOL

Regression of covariate factors on ethambutol Cmax

The peak concentration was transformed by taking the square root in order to satisfy the assumptions of the linear regression model. Changes in haemoglobin, albumin and HIV-infection status within the cohort explained 26% of the variability in the peak concentrations (table 36). HIV-infection was associated with reduced peak ethambutol levels. Lower albumin and higher haemoglobin concentrations were associated with higher Cmax values. The magnitude of the associations could not be assessed because of the square root transformation.

Table 36: Linear regression model of covariate factors on the square root of Cmax

Source	SS	df	MS	Number of obs = 128
Model	5.3593	3	1.7864	F(3, 124) = 14.43
Residual	15.3543	124	.1238	Prob > F = 0
Total	20.7136	127	.1631	R-squared = 0.2587
				Adj R-squared = 0.2408
Sqrt (cmax)				Root MSE = 0.3519

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
HIV-infection	0.3461	0.1073	3.23	0.002	-0.5585	-0.1338
Albumin	0.0396	0.0073	5.46	0.000	-0.0540	-0.0252
haemoglobin	0.1032	0.0233	4.44	0.000	0.0572	0.1492
Constant	2.4009	0.2368	10.14	0.000	1.9323	2.8695

Regression of covariate factors on ethambutol AUCt

The linear regression model (Table 37) of covariate factors on AUCt explained 38% of the variability associated with AUCt. HIV-infection was associated with a 5.88 mg.hr.l^{-1} (approximately 29%) reduction in the AUCt. Patients previously treated for tuberculosis also had AUCt values approximately 16% lower than those receiving treatment for the first time. The ethambutol dose per kilogram of body weight was independently associated with a reduction of 0.34 mg.hr.l^{-1} in AUCt for each 1 mg/kg decrease. As for the Cmax values, higher haemoglobin and lower albumin levels were associated with greater AUCt. Gamma-glutamyl transferase and age were also positively associated with the AUCt.

Table 37: Linear regression model of covariate factors on AUCt

Source	SS	df	MS	Number of obs = 125
Model	2032.583	7	290.3690	F(7, 117) = 10.4
Residual	3265.733	117	27.9122	Prob > F = 0
Total	5298.316	124	42.7284	R-squared = 0.3836
				Adj R-squared = 0.3468
AUCt				Root MSE = 5.2832

	Coef.	Std. Err.	t	P>|t|	[95% Conf. - Interval]	
Dose/kg	0.3366	0.1138	2.96	0.004	0.1112	0.5621
age	0.1059	0.0418	2.54	0.013	0.0232	0.1885
treatment category	-3.2256	1.0666	-3.02	0.003	-5.3379	-1.1133
HIV-infection	-5.8811	1.8164	-3.24	0.002	-9.4784	-2.2838
Albumin	-0.5167	0.1145	-4.51	0.000	-0.7435	-0.2898
γ-GT	0.0268	0.0116	2.30	0.023	0.0037	0.0498
Haemoglobin	1.4202	0.3530	4.02	0.000	0.7212	2.1192
constant	0.5469	5.7101	1.85	0.067	-0.7630	21.8567

Regression of covariate factors on ethambutol AUCt

Like AUCt, the AUC extrapolated to infinity was lower in those infected with HIV, was negatively associated with albumin concentration, and positively associated with γ-GT level and age. Although the association with age is weak (p=0.140), the covariate was significant (p=0.002) in a model excluding 7 potentially influential observations: it is therefore assumed to be an important covariate. Conversely AUCt was not significantly associated with dose/kg, treatment category or haemoglobin; and increased creatinine levels were associated with higher AUCt values. As square root transformation of AUCt was necessary to satisfy the assumptions of the model, estimation of the magnitude of covariate associations was not possible (table 38).

Table 38: Linear regression model of covariate factors on AUCt

Source	SS	df	MS	Number of obs = 120
Model	21.4085	5	4.2817	F(5, 114) = 8.13
Residual	60.0516	114	.5268	Prob > F = 0
Total	81.4601	119	.6845	R-squared = 0.2628
				Adj R-squared = 0.2305
Sqrt (AUCt)				Root MSE = 0.7258

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
HIV-infection	-0.9834	0.2463	-3.99	0.000	-1.4714	-0.4954
Albumin	-0.0249	0.0130	-1.92	0.058	-0.0506	0.0008
γ-GT	0.0046	0.0018	2.62	0.010	0.0011	0.0081
Creatinine	0.0141	0.0039	3.59	0.000	0.0063	0.0219
Age	0.0088	0.0059	1.49	0.140	-0.0029	0.0206
Constant	4.3416	0.6094	7.12	0.000	3.1344	5.5487

Regression of covariate factors on ethambutol elimination rate constant and half-life

The covariate factors entered into the linear regression models had little impact on the rate of elimination or the half-life of ethambutol. Log transformation of the pharmacokinetic measures was necessary to satisfy the assumptions of the weak models (tables 39 and 40) and total protein was the only covariate associated (albeit weakly) with the transformed measures of rate of elimination. For each 1 g/l increase in the total protein, the half-life decreased by 0.7% and the elimination rate constant increased by 0.7%.

Table 39: Linear regression model of covariate factors on ln(half life)

Source	SS	df	MS			Number of obs = 123
Model	0.4387	1	.4387			F(1, 121) = 3.59
Residual	14.8037	121	.1223			Prob > F = 0.0607
Total	15.2424	122	.1249			R-squared = 0.0288
						Adj R-squared = 0.0208
Ln (half-life)						Root MSE = .34988
	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Total protein	-0.0071	.0037	-1.89	0.061	-0.0144	0.0003
_cons	1.5025	.2984	5.04	0.000	0.9118	2.0932

Table 40: Linear regression model of covariate factors on ln(ke)

Source	SS	df	MS			Number of obs = 123
Model	0.4387	1	.4387			F(1, 121) = 3.59
Residual	14.8044	121	.1224			Prob > F = 0.0607
Total	15.2431	122	.1249			R-squared = 0.0288
						Adj R-squared = 0.0208
Ln (ke)						Root MSE = .34988
	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Total protein	0.0071	.0037	1.89	0.061	-0.0003	0.0144
_cons	-1.8690	.2984	6.26	0.000	-2.4597	-1.2783

Regression of covariate factors on ethambutol Tmax

The linear regression model (Table 41) described 24% of the variability associated with the time taken to reach maximal ethambutol concentrations. Smoking status, sex and haemoglobin contributed significantly to the model when 10 potentially influential observations were removed. However, when all observations were included, smoking status, haemoglobin and albumin were not significantly associated with Tmax. Increased ethambutol dose/kg, total protein concentrations, bilirubin levels and AST levels were associated with delayed peak concentrations. Males achieved peak concentrations

approximately 18 minutes later than females. Small decreases in Tmax were associated with increased γ-GT values.

Table 41: Linear regression model of covariate factors on Tmax

Source	SS	df	MS	Number of obs = 126
Model	29.3874	9	3.2653	F(9, 116) = 4.76
Residual	90.9869	116	.7844	Prob > F = 0.0001
Total	120.374	125	.9630	R-squared = 0.2441
				Adj R-squared = 0.1955
				Root MSE = 0.8857

Tmax

	Coef.	Std. Err.	t	P>|t|	[95% Conf. Interval]	
Ethambutol dose/kg	0.0535	0.0185	2.88	0.005	0.0168	0.0902
Sex	0.3062	0.1733	1.77	0.080	-0.0371	0.6495
Smoking	0.1295	0.1806	0.72	0.475	-0.4872	0.2281
Total protein	0.0272	0.0115	2.38	0.019	0.0045	0.0499
Albumin	-0.0215	0.0193	-1.12	0.267	-0.0597	0.0167
Billirubin	0.0436	0.0210	2.07	0.040	0.0020	0.0852
AST	0.0242	0.0111	2.19	0.030	0.0023	0.0461
γ-GT	-0.0047	.0022	-2.11	0.037	-0.0090	-0.0003
haemoglobin	-0.0805	.0632	-1.27	0.205	-0.2056	0.0446
Constant	0.3949	.1314	0.35	0.728	-1.8459	2.6357

Discussion of covariate factor determinants of ethambutol PK measures

Patient and treatment factor effects on the PK measures of ethambutol are summarized in table 42.

The low ethambutol levels have been noted previously in HIV-infected tuberculosis patients[93,55] in comparison to reference ranges. This study demonstrated marked reductions of Cmax, AUCt and AUCi in co-infected patients in comparison to tuberculosis patients without HIV-infection. The reductions in AUCi associated with HIV-infection were approximately 29%. Furthermore, the effect of HIV-infection was statistically significant for Cmax and AUCi, without adjustment for other covariates (figure 14). There was no obvious trend in ethambutol levels related to CD4+ counts in the small sample of HIV infected patients.

In a pattern similar to that for rifampicin, increased albumin was associated with reductions in Cmax, AUCt and AUCi. Conversely, increased haemoglobin concentrations, were associated with elevated measures of bioavailability. The improved bioavailability in patients with higher haemoglobin concentrations was only significant when adjusted for the effects of other covariates in the regression models. The negative association of Cmax, AUCt and AUCi with albumin is stronger, and suggests that

debilitated patients have higher ethambutol concentrations. Age, γ-GT, ethambutol dose/kg and treatment category were also associated with increased AUC values.

Table 42: Summary of the covariate factors to influencing ethambutol pharmacokinetic measures, as determined by linear regression analyses. Only those variables contributing to the final models are included.

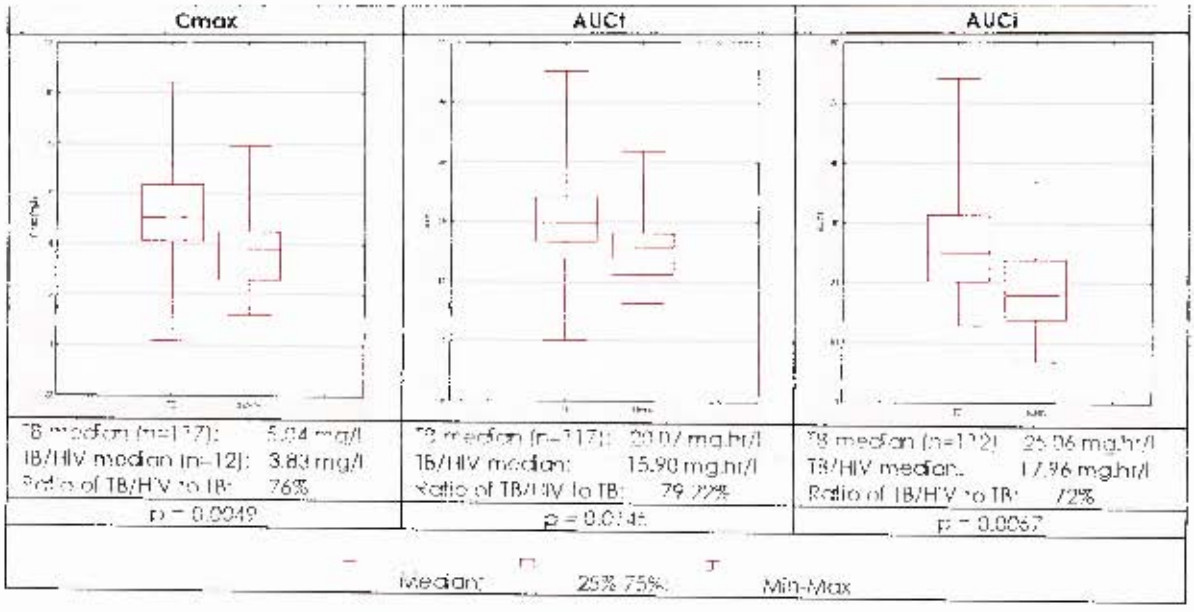
Ethambutol						
PK measure	Cmax	AUC	AUC	Ke	T1/2	Tmax
Transformation	sqrt	-	sqrt	ln	ln	-
R-squared for model	0.25	0.38	0.25	0.03	0.03	0.24
Covariate factors:						
Age (years)	↔	↑	↑	↔	↔	↔
Sex (female=0; male=1)	↔	↔	↔	↔	↔	↑ (0.060)
Smoking[1] (no=0; yes=1)	↔	↔	↔	↔	↔	↔
Alcohol[2] (no=0; yes=1)	↔	↔	↔	↔	↔	↔
Treatment category[3] ('new'=0; 'retreatment'=1)	↔	↑	↔	↔	↔	↔
ethambutol dose/kg	↔	↑	↔	↔	↔	↑
BMI (kg/m²)	↔	↔	↔	↔	↔	↔
Total protein (g/l)	↔	↔	↔	↑ (0.061)	↑ (0.061)	↑
Albumin (g/l)	↓	↑	↓ (0.060)	↔	↔	↔
Total bilirubin (µmol/l)	↔	↔	↔	↔	↔	↑
AST (units/l)	↔	↔	↔	↔	↔	↑
ALT (units/l)	↔	↔	↔	↔	↔	↔
ALP (units/l)	↔	↔	↔	↔	↔	↔
γ-GT (units/l)	↔	↑	↑	↔	↔	↓
Urea (mmol/l)	↔	↔	↔	↔	↔	↔
Creatinine (µmol/l)	↔	↔	↔	↔	↔	↔
Haemoglobin (g/dl)	↑	↑	↑	↔	↔	↔
MCV (fl)	↔	↔	↔	↔	↔	↔
WCC (10^9/l)	↔	↔	↔	↔	↔	↔
Platelets (10^9/l)	↔	↔	↔	↔	↔	↔
ESR (mm)	↔	↔	↔	↔	↔	↔
HIV antibodies (Elisa negative=0; positive=1)	↕	↕	↕	↔	↔	↔
CD4(cells/mL)	↔	↔	↔	↔	↔	↔

↑: the variable contributed significantly to the model describing the PK measure; an increase in the relevant variable was associated with an increase in the value of the PK measure. P values > 0.05 are specified.
↓: the variable contributed significantly to the model describing the PK measure; a decrease in the relevant variable was associated with a smaller value for the PK measure. P values > 0.05 are specified.
↔: no significant contribution to the regression model describing the PK measure
[1] history of smoking in the year prior to admission to Brewelskloof Hospital
[2] history of regular alcohol consumption in the year prior to admission to Brewelskloof Hospital
[3] patients in 'new' category had not received more than 1 month of drug treatment prior to admission; patients in the 'retreatment' category had previously received antituberculosis drugs for one month or longer.

Male sex, bigger doses, and increased plasma protein, total bilirubin and AST levels were associated with delayed peak concentrations. Increased γ-GT levels were associated with more rapid absorption.

Although ethambutol is eliminated by renal mechanisms, neither urea, nor, creatinine levels were significantly associated with the PK measures.

Figure 14: The effect of HIV-infection (right-hand side) on ethambutol levels. The Kruskal-Wallis test was used to determine the significance of the differences.



DISCUSSION

While the linear and logistic regression models were successful in describing some important covariate effects, there was considerable residual variability. Unexplained variation can be attributed to determinants not measured and experimental error. The former could potentially include formulation details (such as raw material and excipient details, conditions of manufacture, conditions of storage, etc.) and numerous unaccounted for patient factors including genetic and environmental determinants of the expression of drug metabolizing enzymes and drug transporting proteins. Data collection procedures were standardized to minimize variation due to experimental error, and blood sampling was frequent to minimize variations in the pharmacokinetic measures due to the experimental design as discussed in the previous chapter.

The study demonstrated that in a country with world class drug regulatory standards for the quality of antituberculosis drugs, considerable variability of rifampicin's bioavailability was conferred by formulation characteristics. Hopefully, the distribution of drug batches that do not comply with national regulations occurs rarely. The reduced bioavailability rifampicin and isoniazid in the fully approved FDCs raises questions about bioequivalence criteria for product registration, ongoing quality assurance procedures for subsequent batches, and the storage conditions required to minimize product deterioration on the shelf.

Even though only 14 patients in this study were co-infected with HIV, none of them had diarrhea and only 4 had CD4+ cell counts less than 200. HIV-infection was an important determinant of the concentrations of the drugs measured. The effects of HIV-infection on the pharmacokinetic measures of rifampicin, isoniazid and pyrazinamide were largely consistent with the findings of a study by Sahai et al.[9] in HIV-infected individuals without tuberculosis. Reports of low antituberculosis drug concentrations in TB/HIV patients in comparison to patients with tuberculosis alone have not been published. The African studies[70,71] designed to address this question were underpowered to do so (without adjustment for important covariates), due to considerable variation in drug concentrations in the patients. Although isolated reports and a case series[58] describe low ethambutol levels in TB/HIV patients, the finding of substantial reductions in ethambutol levels in TB/HIV patients in comparison to patients with tuberculosis alone has not been published. The finding is of particular interest in the light of the increased relapse rates

reported in HIV-infected patients receiving rifampicin sparing regimens with isoniazid and ethambutol in the continuation phase[25]. This study was underpowered to assess the importance of the stage of HIV infection on the drug concentrations.

Males had reduced concentrations of rifampicin, isoniazid and pyrazinamide compared to females. Similarly, Van Crevel et al.[45] noted higher rifampicin levels in women. The sex-based mechanism for the differences is not known, however, the concentrations of drugs that are substrates of p-glycoprotein and CYP3A4 tend to be higher in females[147].

Experiments in cell lines, mice and humans suggest that variability in the P-glycoprotein expression is a major determinant of rifampicin concentrations. A study designed to determine the impact of p-glycoprotein on the rifampicin-inducible expression of CYP3A4 found that rifampicin accumulation is limited by human MDR1-encoded P-glycoprotein in the cell line LS 180 and its derivative clones[105]. Further, they demonstrated that plasma and liver concentrations of rifampicin were several times higher in mdr1a(-/-) vs. mdr1a(+/+) mice given doses of rifampicin by oral gavage (table 43)[105]. The induction of CYP3A was dose dependent: significantly higher levels of CYP3A proteins were detected between in the mdr (-/-) mice when doses of 3 and 5 mg/kg were administered. Thus, the induction of CYP3A seems to depend on the level of rifampicin that the enzyme producing cells are exposed to. While CYP3A does not appear to metabolize rifampicin, the effect may have important implications for drug-drug interactions. Greiner et al. showed a 3.5±2.1 fold increase in p-glycoprotein expression in duodenal biopsies from 8 human subjects after rifampicin treatment [148].

Table 43: Rifampicin levels in liver and plasma from female mdr1a (+/+) and (-/-) mice 24 hour after oral gavage of a single dose of 1.5, 3 or 5 mg/kg. Adapted from Schuetz E, Schinkel A, Relling M, Schuetz J. P-glycoprotein: A major determinant of rifampicin-inducible expression of cytochrome P4503A in mice and humans. Proc. Natl. Acad. Sci. USA 1996; 93: 4001-4005.

Rifampicin dose (mg/kg)	Genotype	Rifampicin* (µg) /500 mg of liver	Ratio (-/-)/(+/+)	Plasma rifampicin levels (µg/ml)	Ratio, (-/-)/(+/+)
1.5	+/+	0.3 ± 0.3	11.3	0.00 ± 0.00	7.0
	-/-	3.4 ± 0.7		0.21 ± 0.14	
3	+/+	1.9 ± 0.5	6.0	0.24 ± 0.20	3.3
	-/-	11.3 ± 4.8		0.79 ± 0.54	
5	+/+	3.8 ± 0.7	2.5	0.57 ± 0.90	3.8
	-/-	9.3 ± 1.4		2.15 ± 1.16	

*3-4 animals / group ± SD.

Measurement of P-glycoprotein expression and activity were beyond the scope of this study. However, study of the genetic polymorphisms associated with P-glycoprotein activity[104,105,147] may provide useful markers of rifampicin levels in the future. Furthermore, environmental and dietary factors influence the activity of P-glycoprotein; and the exposure of patients in this evaluation to these factors was not studied in detail. For example: Piperine, a major constituent of black pepper, inhibits P-glycoprotein in Caco-2 cell cultures[119]. This may explain the approximately 2-fold increase in the plasma levels of rifampicin associated with piperine ingestion in a study published by Zutshi et al in 1985 [118].

V

DETERMINANTS OF TREATMENT RESPONSE

Complex interactions between patient, pathogen and treatment interact to determine treatment response. In this chapter selected cytokine and cellular markers of immunity in the study population, and the susceptibility of *Mycobacterium tuberculosis* isolates cultured from the sputum of a subgroup of study patients are presented. Following this, early and late treatment responses in the study group are summarized and regression models are described for the patient factors (including correlates of immunity, demographic, clinical and laboratory characteristics) and treatment factors (including antituberculosis drug levels and formulation factors) on the markers of treatment response.

Immune response to *Mycobacterium tuberculosis*

Immunity to *Mycobacterium tuberculosis* is conferred predominantly by the cellular immune system. Macrophages are assisted by CD4+ T cells, CD8+ T cells, γδ+ T cells and αβ+ T cells to constrain infection[50].

CD4+ T cells secrete cytokines to enhance macrophage function and regulate the IL-2-mediated expansion of γδ and CD8+ T cell lines. Most CD4+ T cells secrete large amounts of interferon- γ (IFN-γ) and variable amounts of interleukin-2 (IL-2), interleukin 4 (IL 4), interleukin-5 (IL-5) and interleukin-10 (IL-10).

Depending on the local cytokine milieu, the presence of immunologically active hormones, the dose and route of antigen exposure, the type of antigen presenting cell to stimulate the T cell and the strength of the T-cell MHC-antigen complex interaction; newly activated T cells polarize into mature to Th1 or Th2 cells, and accordingly a predominantly type-1 or type-2 response tends to be mounted in response to infections[50]. A predominantly type 1 response is protective against tuberculosis and is characterized by the production of IL-2, IFN-γ and TNF-α effecting cell-mediated immunity with opsonizing antibody production and activation of macrophage-mediated mycobacterial elimination. A type-2 response is associated with IL-4, IL-5, IL-9, IL-10 and IL-13 production with expansion of humoral immunity and inhibition of inflammation.

Healthy individuals with strong tuberculin skin test reactions have immune responses to intracellular bacterial pathogens characterized by IFN-γ production and cytotoxic CD4+ T cell function. In most patients with active pulmonary tuberculosis diminished CD4+ T cell function (with decreased proliferative and IFN-γ responses in peripheral blood to mycobacterial antigen stimulation) is

observed at the time of diagnosis; an immune profile consistent with a type-2 response prevails. The phenomenon is thought to be a consequence of macrophage secretion of cytokines such as TGF-β and IL-10. After 1 month of antituberculosis treatment, IFN- γ production shows recovery in the majority[151]. However, for reasons poorly understood, some patients remain anergic even after the completion of the treatment period[152,153,154].

In this study, selected cellular and cytokine markers of immunity were measured in 112 (79%) of the study patients after 2 months of in-patient treatment for tuberculosis. The results are summarized in figure 1.

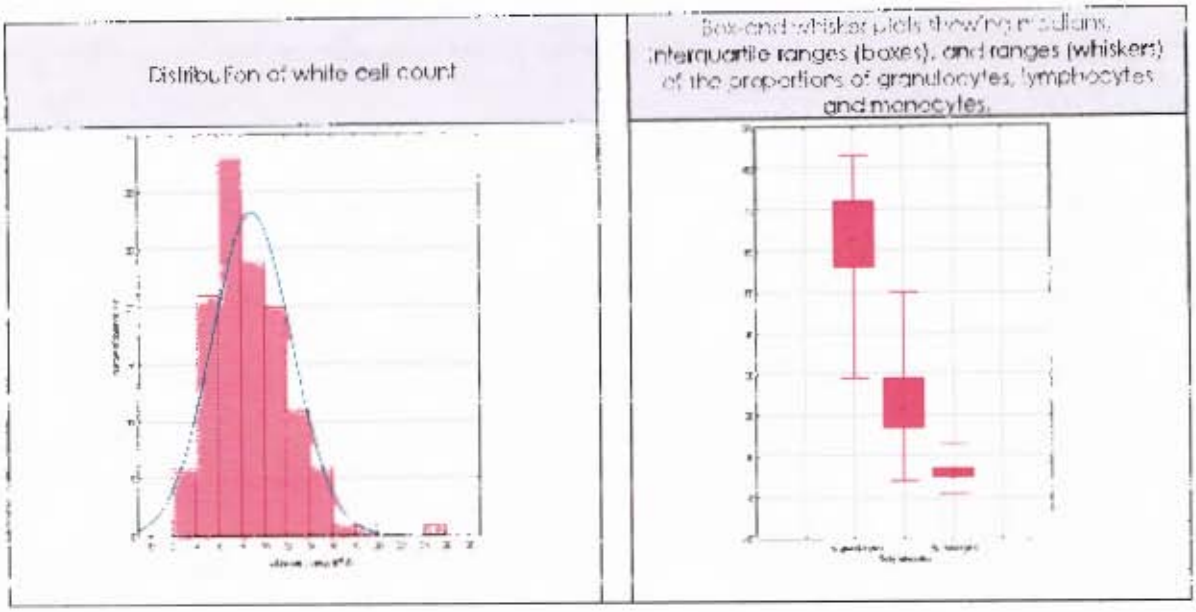
The univariate associations of patient demographic and clinical features, with the immune markers are summarized in table 1.

Increasing age was associated with a tendency to declining total lymphocyte counts, plasma levels of IFN- γ, decreased plasma levels of IL-10, and increased whole blood IL-10 responses to PHA stimulation. Males had lower Plasma IFN- γ (median 33 vs. 73 pg/ml) and higher PHA stimulated responses of IFN-γ (median 247 vs. 73 pg/ml) and IL-10 (median 159 vs. 90 pg/l) than females. Sex hormones influence the polarization of T cell responses (estrogens and progestins inhibit IL-12 and IFN-γ secretion from antigen-presenting cells, while stimulation the production of IL-4, IL-10 and IL-13; while the testosterone derivative DHEA facilitates IL-2 secretion and the establishment of Th1 clones[154] and might, in part, explain the gender differences in the immune response.

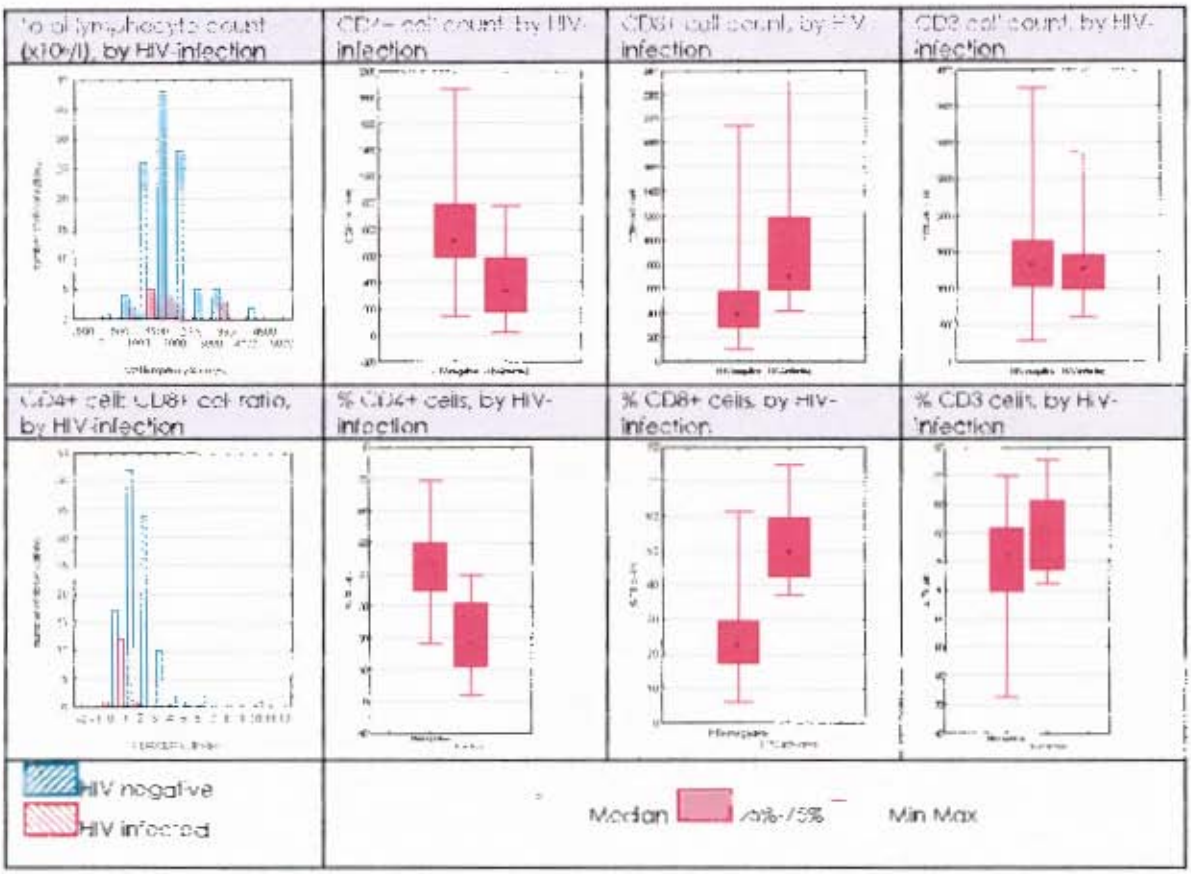
A history of smoking in the year prior to admission affected a number of immune markers. The median total lymphocyte count (1.754 vs. 1.642 x 10^9/l), CD4+ cell count (0.748 vs. 0.533 x 10^9/l), CD3 cell count (1.393 vs. 1.198 x 10^9/l), CD4+ : CD8+ cell ratio (2.0 vs. 1.3), PHA-stimulated IL-10 (172 vs. 93 pg/ml), and PPD-stimulated IFN-γ (1616 vs. 703 pg/ml) were higher in smokers. The increased CD4+ cell count in the peripheral circulation is in keeping with the literature [69,156]. Those with a history of alcohol consumption in the year before admission had higher median CD4+ cell counts (0.760 vs. 0.652 x 10^9/l), CD3 cell counts (1.388 vs. 1.212 x 10^9/l) and PHA-stimulated IL-5 responses (45 vs. 6 pg/ml). As histories of smoking and alcohol use were highly correlated (Chi² p= 0.000) colinearity could partly explain the findings.

Figure 1: Summary of the correlates of immunity in 112 study patients.

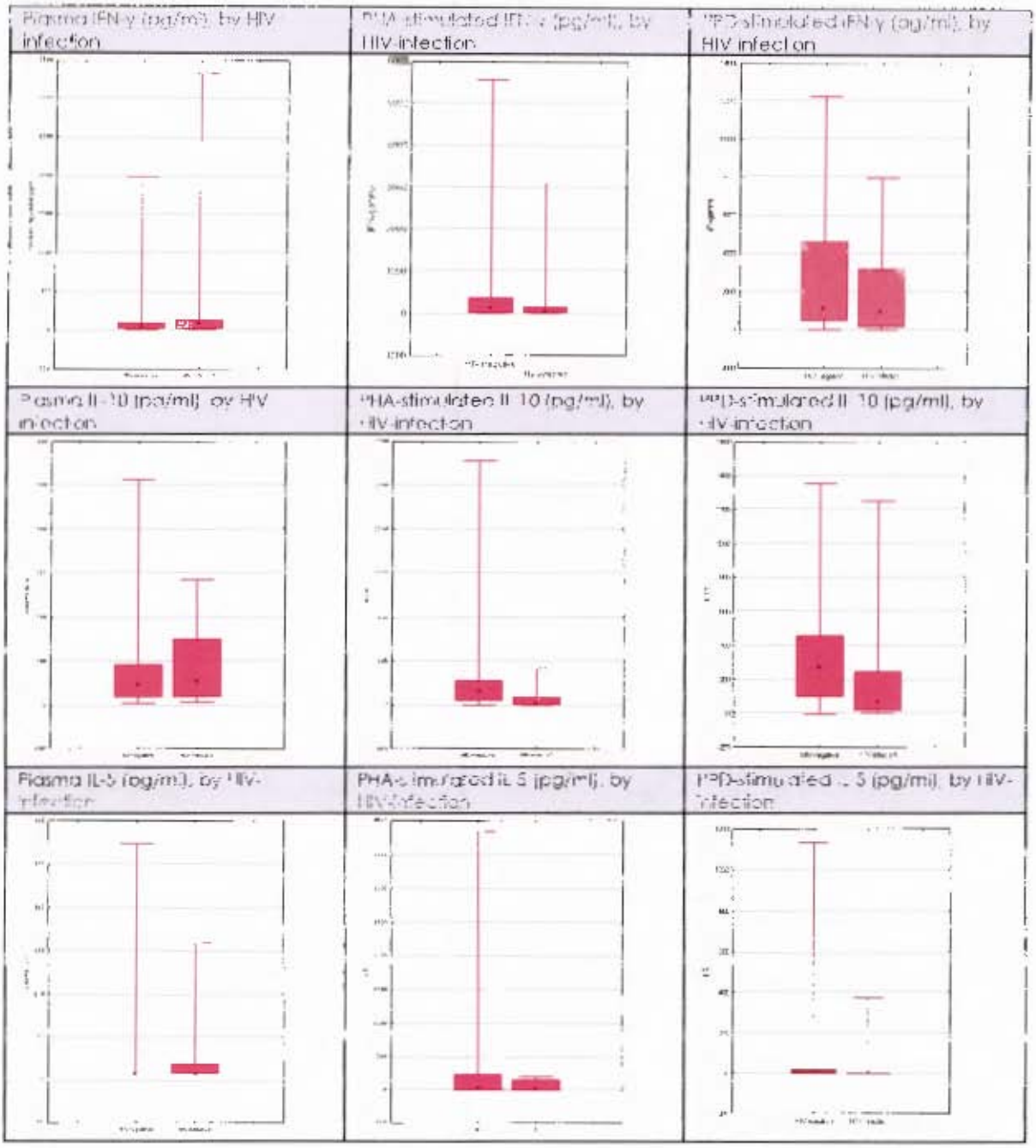
A: The distribution of White cell count (left); and box and whisker plots of the granulocyte, lymphocyte and monocyte percentages.



B: Histograms of the total lymphocyte counts and the CD4+ cell: CD8+ cell ratios; and box-and-whisker plots of the CD4+, CD8+ and CD3 cell counts and their percentages, in HIV-infected (n=14) and uninfected patients.



C: Box and whisker plots of IFN-γ, IL-10 and IL-5, in plasma, and in response to PHA and PPD stimulation, respectively, in HIV infected (n=14) and uninfected patients.



Body mass index was significantly negatively correlated with leukocyte count, granulocyte count and CD3 cell proportion, and significantly positively correlated with lymphocyte count, PHA-stimulated IFN-γ and IL-5 responses, and PPD stimulated IFN-γ responses.

HIV-infected patients had lower median white cell counts (6.05 vs. 8.50 x 10⁹/l), fewer CD4+ cells (median 0.333 vs. 0.718 x 10⁹/l), and lower CD4+cell: CD8+ cell ratios (median 0.4 vs. 2.0), but higher median CD8+ cell counts (0.701 vs. 0.375 x 10⁹/l). In addition, the whole blood IL-10 responses to PHA- and PPD- stimulation were significantly reduced (with medians of 14 vs. 151 pg/ml; and 68 vs. 264 pg/ml, respectively).

Table 1: Matrix displaying the associations of patient characteristics and selected markers of immunity. Spearman's rank correlations (rho, followed by p-value) were used for continuous variables; the probabilities were determined using Kruskal-Wallis (K-W) test for correlations between 2 groups within continuous variables by categorical patient variables. Due to the large number of comparisons within the same patient sample, some of the weaker correlations might be chance findings.

$p < 0.050$ $0.050 \leq p < 0.100$

Patient demographic characteristics vs. immunological profiles		Age		Sex		smoking		alcohol		Transmission category		BMI		HIV-infection	
WCC		.125 .166		K-W: .827		K-W: .083		K-W: .094		K-W: .075		-.339 .000		K-W: 0.4	
% granulocytes		.036 .694		K-W: .627		K-W: .948		K-W: .777		K-W: .146		-.159 .020		K-W: .189	
% lymphocytes		.006 .933		K-W: .952		K-W: .611		K-W: .728		K-W: .058		.235 .001		K-W: .075	
% monocytes		.039 .521		K-W: .191		K-W: .869		K-W: .905		K-W: .380		.026 .773		K-W: .619	
total lymphocyte count		-.152 .062		K-W: .512		K-W: .52		K-W: .45		K-W: .697		-.010 .910		K-W: .171	
CD4 cell count	% CD4+ cells	-.011 .905	.112 .217	K-W: .371	K-W: .401	K-W: .000	K-W: 0u1	K-W: .015	K-W: .039	K-W: .462	K-W: .754	-.089 .445	-.079 .421	K-W: .000	K-W: .000
CD8 cell count	% CD8+ cells	.116 .201	.025 .784	K-W: .441	K-W: .697	K-W: .227	K-W: .092	K-W: .278	K-W: .447	K-W: .380	K-W: .497	-.075 .466	-.062 .555	K-W: .065	K-W: .020
CD3 cell count	% CD3+ cells	-.120 .163	.068 .453	K-W: .664	K-W: .839	K-W: .009	K-W: .2 4	K-W: .023	K-W: .218	K-W: .752	K-W: .455	-.184 .477	-.177 .049	K-W: .299	K-W: .053
CD4:CD8 cell ratio		.039 .554		K-W: .679		K-W: .007		K-W: .199		K-W: .531		.026 .775		K-W: .000	
IFN-gamma	Plasma	.163 .066		K-W: .005		K-W: .123		K-W: .460		K-W: .969		.011 .905		K-W: .698	
	PHA	.104 .276		K-W: .006		K-W: .082		K-W: .010		K-W: .107		.227 0.6		K-W: .351	
	PPD	.072 .443		K-W: .087		K-W: .067		K-W: .846		K-W: .528		.227 .016		K-W: .062	
IL-10	Plasma	-.268 .004		K-W: .604		K-W: .096		K-W: .007		K-W: .751		.054 .573		K-W: .619	
	PHA	.162 .089		K-W: .061		K-W: .016		K-W: .094		K-W: .783		.041 .668		K-W: .001	
	PPD	.065 .442		K-W: .390		K-W: .131		K-W: .139		K-W: .057		-.015 .873		K-W: .029	
IL-5	Plasma	.008 .936		K-W: .783		K-W: .578		K-W: .834		K-W: .695		-.101 .290		K-W: .221	
	PHA	.106 .278		K-W: .348		K-W: .071		K-W: .017		K-W: .094		.196 .041		K-W: .254	
	PPD	.097 .321		K-W: .400		K-W: .405		K-W: .741		K-W: .564		-.073 .486		K-W: .226	

Susceptibility of *Mycobacterium tuberculosis* isolates to rifampicin and isoniazid

The minimum inhibitory concentrations (MICs) of rifampicin and isoniazid were determined for the *Mycobacterium tuberculosis* strains cultured from the sputum of 36 patients (25%), which was

collected on their admission to Brewelskloof Hospital. The culture specimens from most patients were lost due to logistical problems; selection bias is not suspected. The MICs were within the expected ranges for susceptible organisms; the median MIC for rifampicin was 0.25 mg/l and that for isoniazid was 0.05 mg/l. The distribution of MICs for each drug is summarized in table 2.

The small number of isolates available for susceptibility determination precluded the inclusion of the mini-MICs to rifampicin and isoniazid in the analyses of factors determining treatment responses. However, as the difference of a single dilution is not meaningful, it can be seen that there was generally narrow range of susceptibilities to the drugs in the isolates sampled.

Table 2: The minimum inhibitory concentrations for rifampicin and isoniazid, of the isolates cultured from the sputum of 36 patients.

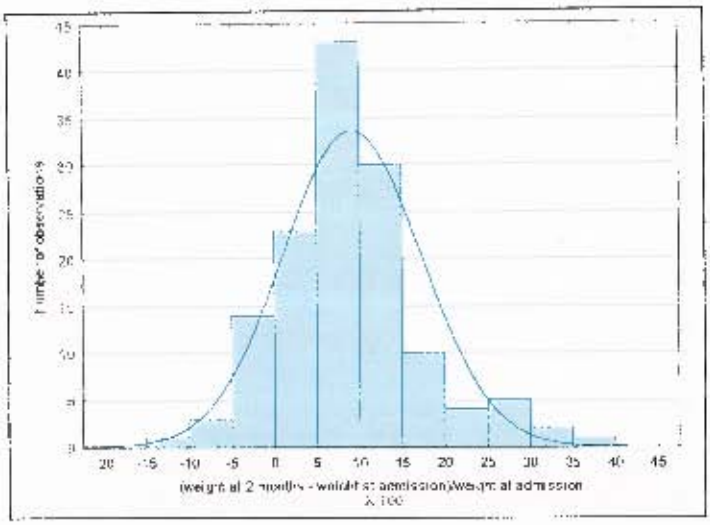
		Isoniazid				
	MIC (mg/l)	0.025	0.05	0.1	0.2	Total
Rifampicin	0.125	6	3	0	0	9
	0.25	2	6	10	1	19
	0.5	1	3	4	0	8
	Total	9	12	4	1	36

PATIENT TREATMENT RESPONSES

Early response

Change in weight over 2 months: The proportional weight gain ([weight at 2 months (kg) - admission weight (kg)] / admission weight (kg) x 100) of patients over 2 months from the time of their admission to Brewelskloof hospital is shown in figure 2. A wide weight response was observed ranging from a 12% weight loss to a 37% weight gain. The median weight change was an 8% gain (interquartile range: 3.87% gain to 13% gain). The change in the weight of patients should not be considered a response specific to antituberculosis treatment, as weight may have been influenced by altered nutritional access during hospitalization.

Figure 2: Histogram of the proportional weight change over 2 months from the time of hospital admission. (Shapiro-Wilks test: p=0.0015)



Sputum direct microscopy and culture at 2 months after admission: The results of the early response based on detection of *Mycobacterium tuberculosis* in the sputum after 2 months of hospitalization are summarized in table 3. Retreatment patients and patients receiving more than 1 month of treatment for the first time ('new' patients) did not have significantly different smear (p=0.185) and culture (p 0.369) results.

Table 3: The results of sputum analysis 2 months after admission to Brewelskloof hospital. The numbers of 'new' (shown in blue) and retreatment (shown in red) patients are indicated in parentheses.

		Early culture result (at 2 months)		
		negative	positive	Total
Direct smear at 2 months	Negative	98 (41 + 57)	10 (2 + 8)	108 (43 + 65)
	Positive	27 (7 + 20)	2 (1 + 1)	29 (8 + 21)
	Total	125 (48 + 77)	12 (3 + 9)	137 (51+86)

Direct smear microscopy and culture results were available for 95.8% (137 of 142) of the study population. The concordance was poor between positive smear and culture results. The proportion of patients with a positive culture (8.8 %) after 2 months of treatment approximated the expected percentage[19]. The high proportion of positive smear results may represent residual nonviable bacilli: the hospitalized group with retreatment patients comprising a majority is likely to have had advanced disease with pulmonary cavitations, this may have resulted in a high proportion of nonviable bacilli after 2 months of treatment. Furthermore, sputum with relatively scant numbers of

viable bacilli (giving positive cultures with negative smear results) might be expected after 2 months of treatment. However, laboratory errors (false positive smears) and logistical shortcomings in specimen storage and transport leading to loss of viability (false negative cultures) could not be excluded.

Late treatment response:

TB register recorded outcome: The treatment outcome as recorded in the clinic based TB registers was available for 126 (88.7%) of study patients. The remaining records could not be traced, either because no record of the patient could be found after they were discharged from Brewelskloof Hospital (12 patients; 5 from the same clinic), or the registers were not available (4 patients from the same clinic where the relevant registers could not be located).

The WHO definitions of treatment outcome (summarized in chapter II) were used in the registers and the results for the study patients are summarized below:

Table 4: Register recorded outcomes for the study cohort and for the region in 2001

	Cured	Treatment completed	Died	Failed	Treatment interrupted	transferred out	TOTAL
			Study cohort				
n	89	11	4	2	19	1	126
%	70.6	8.7	3.2	1.6	15.1	0.8	100
			ostland-Overberg regional statistics for 2001				
%	74	8	2.2	1.8	17		

[1] Statistics from the Department of Health regional office in Worcester

Although they comprised a group of patients referred to hospital for the intensive treatment phase, the register recorded outcomes of the study cohort were similar to those for the entire region (the regional results for 2001 are shown in table 4 for comparison).

For the analysis of factors with a bearing on the register recorded outcome, the outcome was dichotomized into successful treatment ('cured' or 'treatment completed') or unsuccessful treatment ('died', 'failed', 'interrupted treatment'). Thus, 100 successful treatment outcomes (80%) and 25 unsuccessful outcomes (20%), comprised the data used in the analysis. The treatment outcome of the patient 'transferred out' of the region was treated as missing data as the result could not be traced.

Follow up from 2 to 24 months: Patients were followed up through inspection of the clinic records until treatment completion; and (for those without treatment failure or death before treatment completion) regular sputum assessments thereafter, until treatment failure, disease relapse or death, for a maximum of 24 months after their admission to Brewelskloof Hospital.

Thirty-one (21.8%) patients were followed up for longer than 18 months, after hospital admission; 25 (17.6%) of them up to 24 months. Twenty-one (14.8%) patients exited the study from 13 to 18 months; 39 (27.5%) exited from 7 to 12 months; and 51 (35.9%) exited before 6 months. The numbers of patients censored (lost to follow-up) before 24 months and the numbers with relapse or death, are summarized below in table 5.

Table 5: Follow-up of 142 patients from 4 to 24 months; summary of patient attrition over time.

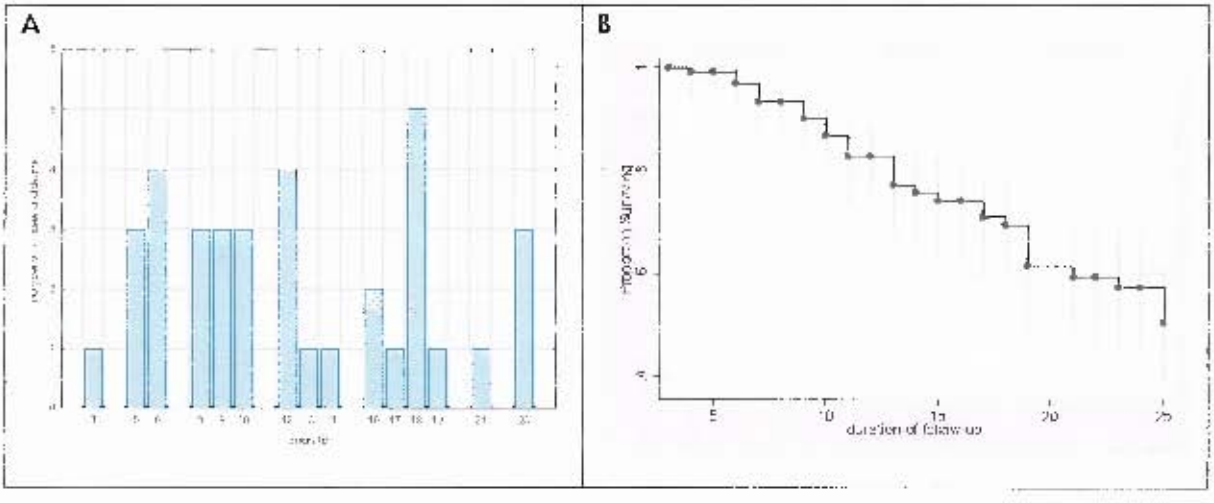
Study period	Number of patients exiting study	Number of patients censored	Number of patients who relapsed or died
2 - 6 months	51	43	8
7 - 12 months	39	26	13
13 - 18 months	21	11	10
19 to 24 months	31	4	5

The large number of early censored observations is not surprising as many patients were from rural locations from where trips to the clinics could be costly and time-consuming. Nine patients, who were censored in the analysis and lost to further follow up, successfully completed treatment within 6 months from the time of their admission to Brewelskloof hospital. These patients had started clinic-based treatment before their admission, and did not return to the clinics for follow up visits. A further 10 observations, which were censored before 6 months, were categorized as having treatment interruption in the TB registers. Six patients could not be traced after their discharge from Brewelskloof Hospital (it is likely that these patients interrupted treatment), and the records of 2 further patients censored before 6 months could not be traced although they were known to the referral clinic. In addition a large number of censored observations from 6 to 12 months can be attributed to patients not returning for any follow-up visits beyond the treatment period, which in many cases corresponded to 6 or 8 months after hospital admission. Should a patients have subsequently suffered a relapse of tuberculosis, it is likely that he/she would have presented to, or been referred to one of the clinics in the region for treatment; and thereby the relapse should have been identified for this study by the register inspection. However, if the patient died before re-registration within the TB clinic system, or if the patient presented to a health care facility outside the regional TB clinic system and were not referred back to the region for treatment; the case would be lost to the study.

Sixteen study patients (44% of the 36 patients with an unfavourable outcome during the 24 month period) died before the end of the observation period. Four were infected with HIV; one HIV-infected patient died during his admission for tuberculosis; the remaining 3 were cured and died later of unknown complications. Of the 12 patients without HIV-infection, 2 died before discharge from the TB hospital; 1 died of massive haemoptysis at home, shortly after discharge; 2 failed treatment; 7 had interrupted treatment; and 2 had relapse after documented cure and treatment completion, respectively; 4 had unknown treatment outcomes, but they died after the treatment period, and the cause of death could not be ascertained from the clinic records (it is likely to have been related to tuberculosis in at least 2 of the cases).

The rate of patients to fail treatment, relapse, or die (figure 3; table 6) was more-or-less constant over the observation period, but with slightly higher rates in the 6 to 12 month period. The higher rates recorded at 6, 12, 18 and 24 months correspond with times when patients were requested to return to the clinics for sputum collections.

Figure 3: The number of cases with treatment failure, relapse or death vs. the time to the event (A), and the proportion of patients without treatment failure, relapse or death over time: Kaplan Meier analysis (B).



The survival analysis predicted a risk of 50% (95% CI: 0.37 - 0.62) for treatment failure, death or relapse at the end of 24 months (figure 3B). The rates of relapse and death might be exaggerated as most of the substantial number of censored individuals are likely to have had a good treatment outcome (most patients developing active tuberculosis should have been detected by the regional TB clinics and the episode recorded in the TB registers). Still, 25% of the 142 patients are known to have failed treatment, relapsed or died within the 24 month period following their admission to the

hospital. The outcome for the region of patients at 24 months after treatment initiation is not known, however the proportion of retreatment patients (being equal to the number of patients receiving treatment for the first time in 2000) is high. Factors contributing poor treatment outcomes during the 24 months might include: a high risk of a poor prognosis in this patient cohort who were admitted to Brewelskloof Hospital for a variety of reasons which might predispose to a poor prognosis (poor treatment adherence, severe or complicated disease, concomitant illness and malnutrition, failure to respond to clinic-based treatment); and this group may be particularly vulnerable to infection with new strains of tuberculosis following treatment in a region with prevalent tuberculosis. Infection with new strains of *Mycobacterium tuberculosis* has been shown in a substantial proportion of patients with disease relapse in the months following treatment in another Western Cape population[157].

Table 6: Kaplan-Meier analysis summary, over the 24 month period following hospital admission.

Time (months)	Total	Failure, relapse or death	Lost	Survival	Error	[95% Conf.	Int.]
1	142	2	11	1	.	.	.
2	131	1	4	0.9924	0.0076	0.9471	0.9989
4	126	0	1	0.9924	0.0076	0.9471	0.9989
5	125	3	12	0.9685	0.0155	0.9184	0.9881
6	110	4	15	0.9333	0.0228	0.8702	0.9661
7	91	0	2	0.9333	0.0228	0.8709	0.9661
8	89	3	6	0.9019	0.0284	0.8289	0.9447
9	80	3	11	0.868	0.0334	0.7859	0.9203
10	66	3	2	0.8286	0.0389	0.736	0.8911
11	61	0	2	0.8286	0.0389	0.736	0.8911
12	59	4	3	0.7724	0.0452	0.6684	0.8474
13	52	1	0	0.7576	0.0468	0.651	0.8356
14	51	1	1	0.7427	0.0481	0.6339	0.8236
15	49	0	3	0.7427	0.0481	0.6339	0.8236
16	46	2	0	0.7104	0.0512	0.5965	0.7975
17	44	1	0	0.6943	0.0525	0.5783	0.7842
18	43	5	7	0.6135	0.0575	0.491	0.715
20	31	1	1	0.5937	0.0589	0.469	0.6984
21	29	0	1	0.5937	0.0589	0.469	0.6984
22	28	1	1	0.5725	0.0605	0.4455	0.6807
23	26	0	1	0.5725	0.0605	0.4455	0.6807
24	25	3	22	0.5038	0.065	0.3712	0.6224

The measures of treatment response: summary

The agreements between the treatment response measures are tabulated below (table 7), and the relationship of each measure to the risk of relapse or death within the 24 month period is illustrated in figure 4.

Table 7: The associations between the treatment response measures.

	2 month weight change	2 month smear microscopy	2 month sputum culture	Register recorded treatment outcome
2 month smear microscopy	Kruskal-Wallis test: p = 0.534			
2 month sputum culture	Kruskal-Wallis test: p = 0.780	Fisher's exact: p = 1.000		
Register recorded treatment outcome	Kruskal-Wallis test: p = 0.116	Fisher's exact: p = 0.593	Fisher's exact: p = 0.655	
2 to 24 month 'survival'	Univariate Cox regression analysis: p=0.604	Log-rank test for equality of survivor functions: p=0.0499	Log-rank test for equality of survivor functions: p=0.380	Log-rank test for equality of survivor functions: p=0.0000

The change in body weight of the patients during the first 2 months of their hospital admission was not significantly correlated with any of the other measures: it was clearly an unreliable predictor of the other early and late response measures in this patient cohort. Many of the patients lived in poverty, and the weight response may have been confounded by improved access to nutrition during the hospital admission.

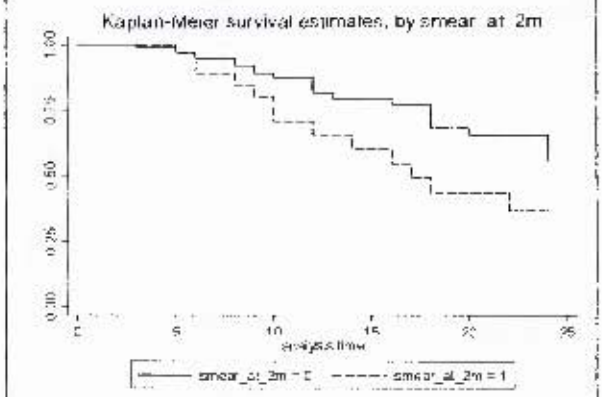
The lack of concordance between the 2 month smear and culture results was discussed previously. The early culture result was not predictive of the outcome over the 24 month period. However, a positive sputum smear after 2 months of in-patient treatment suggested a worse prognosis during the 24 month period of observation (p = 0.050). Positive smear microscopy after at least 2 months of treatment might indicate more extensive cavitatory disease, which is associated with a worse prognosis[43,45]. The number of patients with positive results for both smear and culture was too small to establish whether they were at greater risk of subsequent treatment failure, relapse or death (figure 4D).

Although neither the 2 month sputum microscopy results, nor the early culture results corresponded to the treatment outcomes recorded in the TB registers, the TB register records were consistent with the survival analysis; as can be expected, patients with treatment failure, treatment interruption, or death according to the TB registers, had significantly increased risks of poor outcomes during the 24 months after admission to Brewelskloof Hospital (p = 0.000).

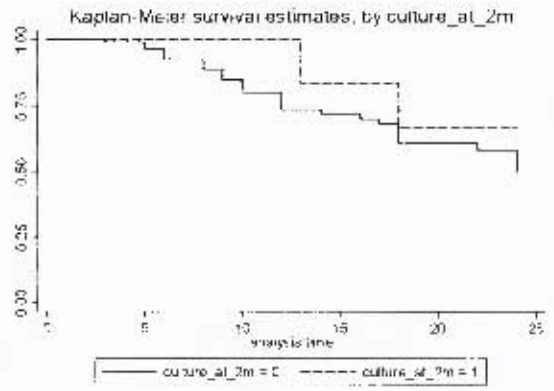
The poor correlations of the early culture result and the survival analysis results, and the register-recorded outcome at the end of treatment and the early sputum markers, are probably, in part, due confounding factors not evaluated in this study (such as poor treatment adherence)[1] but the possibilities of false positive sputum smears and of loss of culture viability during, or while awaiting, transfer to the Green Point laboratory, should be explored.

Figure 4. The survival estimates (from 2 to 24 months), by the early sputum results (A, B, D) and the treatment outcome recorded in the TB registers (C).

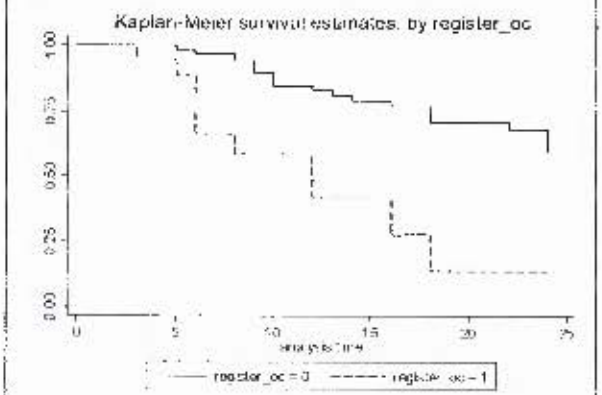
A: Survival estimates by smear microscopy at 2 months (dashed line indicates patients with positive 2-month smear).



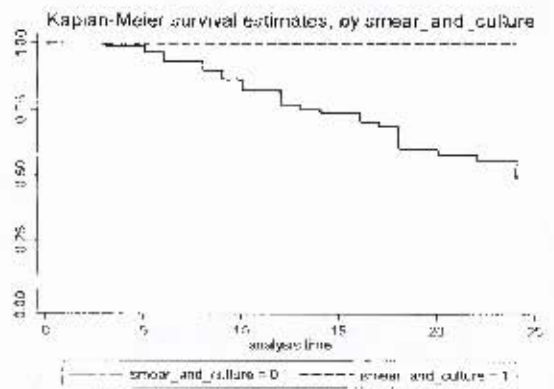
B: Survival estimates by early sputum culture (dashed line indicates patients with a positive early culture result).



C: Survival estimates by the TB register outcome (dashed line indicates treatment failure, interruption or death).



D: Survival estimates in those with positive results for both smear and early culture (dashes) vs. the rest of the patient cohort; Log rank test for equality of survivor functions: p = 0.390



DETERMINANTS OF TREATMENT RESPONSE

The univariate patient, treatment and pathogen factor associations with the treatment response measures are summarized in table 8. The MICs of the *Mycobacterium tuberculosis* isolates to rifampicin and isoniazid were not included in the multivariate regression analyses, as the small number of MIC observations would curtail the sample sizes, thus limiting the power of the analyses. All other covariates listed in table 5 were evaluated in the multivariate analyses described in the sections to follow. The standardized procedure outlined in the methods section was used for variable selection. As the Cmax, AUCt and AUCi for each drug were highly correlated with one another, only the pharmacokinetic measure with the strongest contribution to the model was included, in order to avoid multi-colinearity.

Table 8. Univariate analyses evaluating the significance of associations between the measures of response and patient, treatment and pathogen factors. Spearman's rank correlations (rho, followed by p-value) were used to determine the correlation between 2 continuous variables; the probabilities were determined using the Kruskall-Wallis rank test (K-W) for comparison of 2 groups (by a categorical variable) within a continuous variable; the Pearson's Chi² (Chi2) test was used to determine the probability of the association between 2 categorical variables; and univariate Cox regression was used to determine the probability of the association of the patient, treatment and pathogen factors with the risk of relapse or death from 2 to 24 months. Due the large number of comparisons within the same patient sample, some of the weaker correlations might be chance findings.

P < 0.050 0.050 ≤ p < 0.100

Outcome vs. Demographic and clinical characteristics	Change in weight	sputum		Registered recorded treatment outcome	2 to 24 month follow up (cox regression hazard ratio; p-value)
		2 month Direct Microscopy	Culture at 2 (or 3) months		
Age	1044 p=.226	k-W: p=. 41	k-W: p=.051	k-W: p=.648	0.985 p=.279
Male sex	K-W: p=.349	Chi2: p=.408	Chi2: p=.140	Chi2: p=.473	1.033 p=0.926
Smoking	K-W: p=.490	Chi2: p=.841	Chi2: p=.080	Chi2: p=.825	2.744 p=.388
Alcoho	K-W: p=.080	Chi2: p=.770	Chi2: p=.164	Ch2: p=.598	1.119 p=.736
[illegible]	K-W: p=.667	Chi2: p=.185	Chi2: p=.282	Chi2: p=.028	761 p=.151
BMI	2084 p=.61.5	k-W: p=.907	k-W: p=.949	k-W: p=.243	0.900 p=.097
Intermediate or fast acetylator type	K-W: p=.665	Chi2: p=.918	Chi2: p=.970	Chi2: p=.958	1.427 p=.531
HIV-infection	K-W: p=.538	Chi2: p=.958	Chi2: p=.571	Ch2: p=.611	1.700 p=.208

Outcome vs. Demographic and clinical characteristics	Change in weight	sputum		Registered recorded treatment outcome	2 to 24 month follow up (cox regression hazard ratio; p-value)
		2 month Direct Microscopy	Culture at 2 (or 3) months		
Urea	.0599 p=.488	k-W: p=.075	k-W: p=.366	k-W: p=.025	0.735 p=.108
Creatinine	.0109 p=.592	k-W: p=.094	k-W: p=.756	k-W: p=.015	0.961 p=.003

Total protein	.1140 p=.186	k-W: p=.850	k-W: p=.230	k-W: p=.983	1.022 p=.311
albumin	.3762 p<.000	k-W: p=.037	k-W: p=.927	k-W: p=.320	0.892 p=.003
ALT	.1891 p=.028	k-W: p=.731	k-W: p=.717	k-W: p=.189	1.001 p=.958
AST	.0377 p=.663	k-W: p=.217	k-W: p=.569	k-W: p=.837	1.000 p=.981
Alkaline phosphatase	-.2985 p=.000	k-W: p=.348	k-W: p=.298	k-W: p=.603	1.004 p=.358
γ-GT	-.1871 p=.029	k-W: p=.333	k-W: p=.256	k-W: p=.617	1.004 p=.193
Total bilirubin	-.1864 p=.330	k-W: p=.167	k-W: p=.122	k-W: p=.026	0.918 p=.208
Haemoglobin	.2023 p=.018	k-W: p=.054	k-W: p=.730	k-W: p=.336	0.927 p=.451
MCV	-.0915 p=.292	k-W: p=.325	k-W: p=.397	k-W: p=.355	1.000 p=.980
WCC	-.0681 p=.462	k-W: p=.005	k-W: p=.098	k-W: p=.934	1.062 p=.147
Platelet count	-.0949 p=.275	k-W: p=.002	k-W: p=.475	k-W: p=.779	1.002 p=.094
ESR	-.3227 p=.000	k-W: p=.035	k-W: p=.597	k-W: p=.373	1.007 p=.093

Outcome vs. marker of immunity		Change in weight		Culture				Rapid recorded treatment outcome		2 to 24 month follow-up Cox regression hazard ratio p-value	
				2 month Direct Microscopy		Culture at 2 (or 3) months					
% granulocytes		-.1234 p=.064		k-W: p=.017		k-W: p=.930		k-W: p=.108		1.040 p=.039	
% lymphocytes		.1430 p=.021		k-W: p=.013		k-W: p=.421		k-W: p=.288		0.977 p=.311	
% monocytes		.0092 p=.921		k-W: p=.107		k-W: p=.372		k-W: p=.538		0.993 p=.227	
Total lymphocyte count		.1099 p=.234		k-W: p=.493		k-W: p=.559		k-W: p=.771		1.000 p=.437	
CD4 cell count	% CD4+ cells	.1089 p=.238	.0611 p=.509	k-W: p=1.00	k-W: p=.137	k-W: p=.452	k-W: p=.946	k-W: p=.367	k-W: p=.116	1.000 p=.782	0.998 p=.854
CD8 cell count	% CD8+ cells	-.367 p=.738	.0663 p=.353	k-W: p=.126	k-W: p=.33	k-W: p=.737	k-W: p=.920	k-W: p=.233	k-W: p=.12	1.000 p=.609	1.005 p=.70
CD3 cell count	% CD3+ cells	-.202 p=.193	.0667 p=.471	k-W: p=.877	k-W: p=.023	k-W: p=.911	k-W: p=.668	k-W: p=.777	k-W: p=.546	1.000 p=.491	1.004 p=.010
CD4:CD8 cell ratio		-.0348 p=.707		k-W: p=.169		k-W: p=.867		k-W: p=.100		1.034 p=.197	
IFI gamma	Plasma	.0827 p=.220		k-W: p=.453		k-W: p=.017		k-W: p=.539		0.998 p=.392	
	PHA	.3418 p=.0003		k-W: p=.020		k-W: p=.532		k-W: p=.653		1.000 p=.932	
	PPD	.2597 p=.009		k-W: p=.815		k-W: p=.908		k-W: p=.441		1.000 p=.009	
IL-10	Plasma	-.2495 p=.035		k-W: p=.152		k-W: p=.975		k-W: p=.172		0.998 p=.343	
	PHA	.2040 p=.035		k-W: p=.593		k-W: p=.809		k-W: p=.174		0.999 p=.404	
	PPD	.0061 p=.953		k-W: p=.316		k-W: p=.686		k-W: p=.589		0.998 p=.055	
IL-5	Plasma	-.2166 p=.075		k-W: p=.682		k-W: p=.726		k-W: p=.427		0.998 p=.734	
	PHA	.2021 p=.040		k-W: p=.700		k-W: p=.458		k-W: p=.797		1.000 p=.585	
	PPD	.1109 p=.269		k-W: p=.659		k-W: p=.345		k-W: p=.455		0.999 p=.208	

Outcome vs. drug factors	Change in weight [1]	culture		Register recorded treatment outcome	2 to 24 month follow-up (cox regression hazard ratio; p-value)
		2 month Direct Microscopy	Culture at 2 (or 3) months		
Rifampicin MIC	.2404 p=.171	K-W: p=.523	K-W: p=.292	K-W: p=.43	0.927 p=.170
isoniazid MIC	-.0834 p=.299	K-W: p=.956	K-W: p=.960	K-W: p=.855	8390 p=.018

Outcome vs. drug factors		Change in weight [1]	culture		Register recorded treatment outcome	2 to 24 month follow-up (cox regression hazard ratio; p-value)
			2 month Direct Microscopy	Culture at 2 (or 3) months		
rifampicin	Cmax	.0928 p=.283	K-W: p=.146	K-W: p=.809	K-W: p=.016	1.014 p=.752
	AUCt	.0502 p=.561	K-W: p=.247	K-W: p=.552	K-W: p=.013	1.002 p=.829
	AUCi	.658 p=.475	K-W: p=.601	K-W: p=.726	K-W: p=.536	0.994 .642
isoniazid	Cmax	.1251 p=.147	K-W: p=.526	K-W: p=.233	K-W: p=.039	1.008 p=.910
	AUCt	.0664 p=.444	K-W: p=.625	K-W: p=.383	K-W: p=.018	0.992 p=.667
	AUCi	.3497 p=.567	K-W: p=.506	K-W: p=.499	K-W: p=.119	0.997 p=.553
pyrazinamide	Cmax	.02441 p=.002	K-W: p=.373	K-W: p=.074	K-W: p=.096	1.003 p=.656
	AUCt	.2770 p=.001	K-W: p=.125	K-W: p=.245	K-W: p=.059	1.002 p=.492
	AUCi	-.2226 p=.015	K-W: p=.100	K-W: p=.695	K-W: p=.207	1.000 p=.952
ethambutol	Cmax	-.2203 p=.014	K-W: p=.349	K-W: p=.026	K-W: p=.663	1.005 p=.339
	AUCt	.1956 p=.053	K-W: p=.424	K-W: p=.054	K-W: p=.465	1.006 p=.794
	AUCi	-.1763 p=.056	K-W: p=.537	K-W: p=.044	K-W: p=.336	1.011 p=.606
rifampicin dose/kg		-.3427 p=.000	K-W: p=.304	K-W: p=.728	K-W: p=.183	1.225 p=.042
isoniazid dose/kg		-.2057 p=.016	K-W: p=.079	K-W: p=.132	K-W: p=.161	1.347 p=.018
Pyrazinamide dose/kg		-.3466 p=.000	K-W: p=.047	K-W: p=.346	K-W: p=.332	1.089 p=.166
Ethambutol dose/kg		-.2993 p=.000	K-W: p=.130	K-W: p=.579	K-W: p=.358	1.021 p=.365
Non-approved rifampicin product		K-W: p=.114	Chi2: p=.930	Chi2: p=.968	Chi2: p=.683	0.640 p=.204
Rifampicin and isoniazid in FDC		K-W: p=.430	Chi2: p=.134	Chi2: p=.728	Chi2: p=.714	2.347 p=.521

Unless otherwise indicated, Spearman's rank correlation; p-value; K-W implies Kruskal-Wallis rank test was applied for categorical variables; p-value quoted.
[1] In this exploratory analysis 4 observations were included as early culture positive if the 3 month culture was positive when the result of the 2 month culture was not known or negative.

<u>Factors associated with weight gain from the time of admission until 2 months thereafter</u>

In order to satisfy the assumptions of the multiple linear regression model (table 9), the square root transformation of the proportional weight gain was used. Patients who lost weight during the 2 month period were assigned a value of 0, as the square root of the negative values could not be

determined. A further limitation of the transformation is that while the direction and statistical significance of the covariate effects on the proportional weight change could be assessed, they could not be quantified.

Patients with lower body mass indices at 2 months had gained proportionately less weight during the preceding 2 months. Higher albumin and lower total protein levels were significantly associated with weight gain over the 2 month period. Albumin levels decline and total protein levels rise as part of the inflammatory response to infection, increased albumin and decreased total protein levels, respectively, may therefore reflect a positive response to treatment. Similarly, higher bilirubin concentrations may be associated with more severe disease (or less disease resolution), and it is not surprising that total serum bilirubin was negatively associated with weight gain over the preceding 2 months.

Table 9: Linear regression model describing the relationship of covariates to the square root of the proportional weight gain over the initial 2 months of hospital admission

Source	SS	df	MS			Number of obs = 124
Model	86.2093	6	14.3682			F(6, 117) = 10.49
Residual	160.2275	117	1.3695			Prob > F = 0
Total	246.4368	123	2.0036			R-squared = 0.3498
						Adj R-squared = 0.3165
Sqrt (% weight gain)						Root MSE = 1.1702
	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Total protein	-0.0311	0.0131	-2.37	0.020	-0.0570	-0.0051
Albumin	0.0848	0.0212	4.01	0.000	0.0429	0.1267
Body mass index	-0.1106	0.0427	2.59	0.011	-0.1952	-0.0259
Ethambutol AUCt	-0.0476	0.0171	-2.78	0.006	-0.0815	-0.0137
Total bilirubin	-0.0583	0.0265	-2.20	0.030	-0.1108	-0.0059
Pyrazinamide dose/kg	-0.0746	0.0232	-3.22	0.002	-0.1206	-0.0287
_cons	8.1926	2.0674	3.96	0.000	4.0981	12.2871

Patients with higher ethambutol levels (as indicated by the AUCt) gained less weight over the 2 month period (figure 5). Conversely, increased ethambutol levels were associated with a greater likelihood of sputum sterilization at 2 months (table 11), suggesting that the drug's effect on weight gain was opposite to the effect on sputum sterilization. Increased drug concentrations might, for instance, be associated with nausea and consequently less weight gain. Patients not receiving ethambutol were not included in the model.

Patients with higher doses of pyrazinamide (adjusted for weight) had smaller weight gains (figure 6). The range of pyrazinamide doses prescribed was wide; from 19.69 to 52.63 mg/kg (median 35.71 mg/kg). Antagonism of the treatment response to the other drugs with higher doses of pyrazinamide due to a pharmacokinetic interaction is unlikely, because neither increased pyrazinamide concentrations, nor, dose/kg was associated with decreased rifampicin, isoniazid or

ethambutol levels (data not shown). Although a pharmacodynamic interaction cannot be excluded, the effect is more likely to be related to the widely recognized side effects of nausea, anorexia and vomiting[4,7,25] that are associated with pyrazinamide. The negative association with weight gain is thus more likely to reflect a direct effect on weight gain, rather than a reduced response of the disease to treatment. The relationship between proportional weight gain and drug dose/kg was essentially the same for those receiving pyrazinamide doses less than 35 mg/kg (the maximum recommended dose[172]) and those receiving larger doses: spearman's rho -0.2697 (p=0.0273) and -0.2692 (p 0.0253), respectively.

Figure 5: The correlation between ethambutol AUCI and proportional weight gain. Spearman's rho: 0.3256 (p=0.0002)

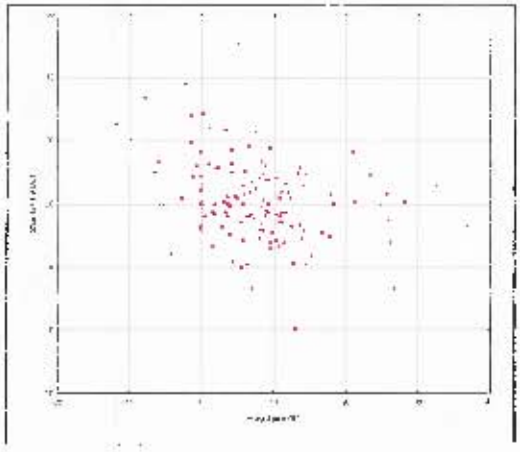
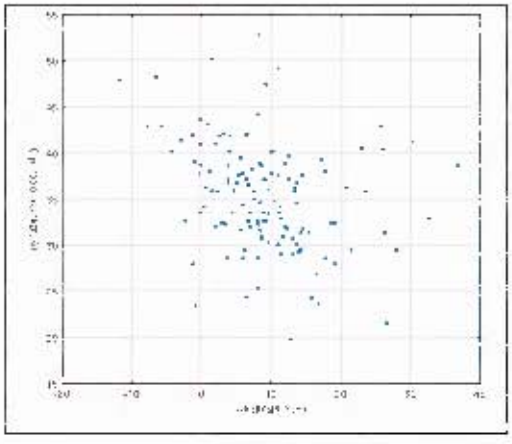


Figure 6: The correlation between pyrazinamide dose/kg (mg/kg) and proportional weight gain. Spearman's rho: -0.3486 (p=0.0003)



Covariates associated with the results of sputum smear microscopy and culture after 2 months of treatment in hospital

Multiple logistic regressions were used to determine the relative risks associated with covariate factors of a positive sputum smear (table 10), and culture (table 11), respectively.

Increasing age was associated with an increased risk of smear and culture positivity at 2 months, even though concordance between the 2 measures was poor. For each year of age the associated risk was 1.04 for a positive smear: a 49% increase in the relative risk was associated with each advancing decade. The risk of a positive culture increased by approximately 10% per year, thus conferring a relative risk of 2.53 for each decade. Thus, in this study, increasing age was strongly associated with a poor response to treatment at 2 months. The association between age and relapse rates has studied elsewhere, but the findings are inconsistent between different studies[58].

For each 1 x 10^9 cells/l increase in the platelet count, the relative risk of a positive 2 month smear increased by 0.38%; this translates into a 21% increase in the relative risk of a positive 2 month smear for each 50 x 10^9 cells/l increase in the platelet count. A risk reduction of 39% for was associated with each 5 g/l increase in albumin (a single unit increase in albumin being associated with a risk reduction of 9.44%). For each 1 x 10^9 cells/l increase in the white cell count there was a 33% increase in the risk of a positive early sputum culture. These findings are consistent with a direct association between the markers of the acute phase response and the persistence of *Mycobacterium tuberculosis* in the sputum.

Table 10: Logistic regression model describing the relative risk associated with covariate factors of a positive sputum smear microscopy at 2 months after hospital admission

Logistic regression Log likelihood = -61.9536					Number of obs = 141 LR chi2(4) = 22.06 Prob > chi2 = 0.0002 Pseudo R2 = 0.1511	
+ sputum smear at 2 months						
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. interval]	
age	1.0407	0.0206	2.02	0.043	1.0012	1.0818
Platelet count	1.0038	0.0015	2.61	0.009	1.0010	1.0067
Rifampicin AUCt	0.9654	0.0155	-2.19	0.028	0.9355	0.9963
albumin	0.9056	0.0415	-2.16	0.031	0.8278	0.9907

A 30% reduction in the risk of a positive 2 month smear was associated with each 10 mg.hr/l increase in the AUCt of rifampicin (each 1 mg.hr/l resulted in a 3.46% risk reduction). The association was not significant without adjustment for the other risk factors (the median rifampicin AUCt for smear negative patients was 20.32 mg.hr/l vs. 18.20 mg.hr/l for those with a positive sputum smear at

2 months; p=0.147). It is interesting, that no relationship of rifampicin levels to the sputum culture result was apparent. This may be related to the reduced power to detect an effect on sputum culture in comparison to direct microscopy (as fewer patients had positive sputum cultures than smears).

On the other hand, the peak concentrations of ethambutol were an important determinant of sputum sterilization at 2 months, but did not have an impact on smear conversion. Patients not receiving ethambutol (because of contraindications) were not included in the model.

Table 1: Logistic regression model describing the relative risk associated with covariate factors of a positive sputum culture at 2 months after hospital admission

Logistic regression					Number of obs = 121	
Log likelihood = -29.6876					LR chi2(3) = 14.91	
					Prob > chi2 = 0.0019	
+ sputum culture at 2 months					Pseudo R2 = 0.2007	
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
age	1.0608	0.03274	2.73	0.006	1.0235	1.1919
Ethambutol Cmax	0.4471	.1394	-2.58	0.010	0.2427	0.8239
White cell count	1.2479	.1378	2.01	0.045	1.0050	1.5496

A 55% reduction in the risk of a positive sputum culture was associated with each 1mg/l increase of ethambutol Cmax. Even without adjustment for the other risk factors, significantly lower ethambutol concentrations were found in those with positive sputum cultures at 2 months (4.14 mg/l vs. 5.06 mg/l; p=0.026). The importance of this finding is supported by the association in this study of HIV-infection with reduced ethambutol concentrations together with recent evidence that HIV-infected patients receiving 8-month rifampicin-sparing regimens with isoniazid and ethambutol in the continuation phase, have worse treatment outcomes than patients without HIV-infection on similar regimens[29]. However, it contradicts growing concerns that the inclusion of ethambutol in antituberculosis regimens may be counterproductive. Jindani et al.[34] demonstrated that the 2 to 14 day activity of multi-drug regimens (measured by the fall in colony form units in the sputum) was antagonized by the inclusion of ethambutol, which appeared to inhibit the sterilizing activity of companion drugs. The role of ethambutol within a treatment regimen may be dose or concentration dependent, and may also depend on the concentrations of the companion drugs. In this regard it is important to note that the doses of ethambutol administered to patients in this study were relatively high in comparison to the 15 mg/kg recommended by most references[62,128], and the concentrations of rifampicin were low in many patients.

Covariates associated with the results of the register-recorded treatment outcome upon completion of treatment

Patients with an unfavourable outcome (treatment interruption, failure or death) comprised substantially treatment interrupters (Table 4), who would be among the observations censored early in the analysis of factors determining the risk of relapse or death up to 24 months. Even though treatment interrupters predominated those with an unfavourable outcome in the TB registers, a logistic regression analysis of risk factors for treatment interruption alone (not shown), found that increased haemoglobin was the only covariate significantly associated with treatment interruption (relative risk 1.82; p=0.012). Patients with higher haemoglobin levels at 2 months after admission might have suffered less severe illness (indeed some may have been admitted to the hospital because of poor adherence to clinic based treatment) or recovered more rapidly on treatment and as a result they might have been less motivated to completed treatment or received less encouragement to complete treatment.

Table 12: Logistic regression model describing the relative risk associated with covariate factors of an unfavorable outcome (treatment interruption, treatment failure or death) according to the clinic based TB registers

Logistic regression Log likelihood = -53.535987 **Register-recorded outcome**					Number of obs = 125 LR chi2(3) = 18.02 Prob > chi2 = 0.0004 Pseudo R2 = 0.1441	
	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
Treatment category (Retreatment)	3.2767	1.8644	2.09	0.037	1.0743	9.9942
Urea	0.5507	0.1599	-2.05	0.040	0.3117	0.9730
Isoniazid Cmax	1.2637	0.1136	2.6	0.009	1.0596	1.5070

Retreatment patients were 3.28 times more likely than 'new' patients to have an unfavourable outcome recorded in the TB registers. Retreatment patients comprised 64% of the study population, and substantial proportion of the patients in the region. Clearly this group of patients is vulnerable to repeated cycles of disease.

Higher urea concentrations were associated with improved TB register outcomes; each increase of 1 mmol/l at 2 months was associated with a risk reduction of 45% of an unfavorable outcome. Low urea concentrations are associated with wasting, and might indicate poor background nutritional status or disease severity; these factors may be associated with reduced immunity, and, or, low socio-economic status, which, in turn, may predispose patients to an unfavorable outcome at the end of the treatment period for a variety of reasons. However, if these reasons are valid, urea

concentration seems to be a better marker for wasting than BMI, which was not included in the model.

Worse outcomes on completion of treatment were associated with higher peak isoniazid concentrations at 2 months after admission to the hospital. Each 1 mg/l increase in the isoniazid concentration was associated with 26% increase in the risk of treatment interruption, treatment failure or death. The median (5th to 95th centile range) Cmax values for isoniazid in each group were 7.62 mg/l (4.13 – 14.06) and 6.04 mg/l (3.42 – 11.50) for those with unfavourable and favourable outcomes, respectively. One explanation for the finding could be concentration dependent isoniazid toxicity, and related reductions in treatment adherence; in this group of patients poor treatment adherence can be expected to be more likely during the continuation phase of treatment, after discharge from the hospital. However, although treatment interrupters comprised a high proportion (76%) of the group with an unfavourable outcome according to the TB registers, isoniazid levels were not significantly associated with treatment interruption per se. The inclusion of isoniazid in regimens containing rifampicin and pyrazinamide, and regimens containing rifampicin, pyrazinamide and moxifloxacin has been associated with reduced sterilizing activity in murine models[59, 60]. The basis for this finding is unknown; a pharmacokinetic interaction between isoniazid and rifampicin has been proposed[59], in this study, while all patients received isoniazid, neither isoniazid levels, nor, dose/kg were associated with decreased rifampicin concentrations (data not shown).

Covariates associated with the risk of treatment failure, disease relapse or death from 2 to 24 months

Immune factors measured at 2 months featured prominently in the regression model describing the covariates associated with the risk of treatment failure, death or relapse up to 24 months.

HIV-infected patients tended to have a risk 2.45 times greater than patients without HIV of a poor outcome during the 24 months (p=0.066). The study was underpowered to establish this relationship with confidence, but the finding is consistent with other studies demonstrating a high mortality amongst HIV-infected patients with tuberculosis[161, 162]. While treatment regimens containing rifampicin throughout are thought to be equally effective in HIV-infected and HIV-uninfected patients, rifampicin sparing regimens are associated with increased relapse rates in HIV-infected cases. However, the very low rifampicin levels demonstrated in this study, in addition to the high

prevalence of tuberculosis in the community might have predisposed immunocompromised patients, in particular, to higher relapse rates.

The whole blood IFN-γ response to PPD stimulation at 2 months was associated with a substantially improved treatment response. The hazard ratio of 0.9996 reflects the effect of a 1 unit increase in the IFN-γ response and translates to a 32% reduction in the relative hazard of treatment failure, death or relapse for every 1000 unit increase in the IFN-γ response to stimulation with PPD. In a univariate analysis, those with IFN-γ responses in the highest quartile (> 4554 pg/ml) had a 92% reduction in the relative hazard of a poor treatment response by 24 months, in comparison to those in the lowest quartile (≤ 447.5 pg/ml).

Table 13: Cox proportional hazards model showing the relative risks of factors associated with a worse treatment outcome

No. of subjects = 110 — Number of observations = 110
No. of failures = 27
Time at risk = 1376

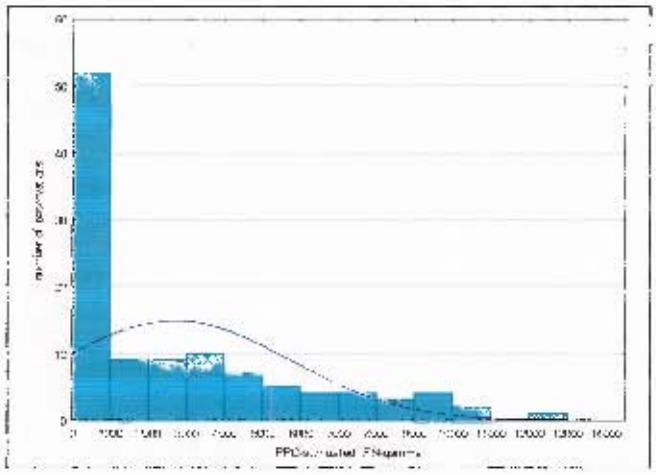
LR chi2(5) = 27.87
Log-likelihood = -91.6217 — Prob > chi2 = 0

	Hazard ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
IFN-γ response to PHA	1.0009	0.0003	2.74	0.006	1.0002	1.001
IFN-γ response to PPD	0.9996	0.0001	-2.94	0.003	0.9993	0.9999
creatinine	0.9509	0.0169	2.83	0.005	0.9184	0.9846
% granulocytes	1.0481	0.0245	2.01	0.045	1.0011	1.0973
HIV-infection	2.4547	1.1981	1.84	0.066	0.9430	6.3893

Hirsch et al found mean (±SD) whole blood IFN-γ responses to PPD stimulation of 316 pg/ml (± 74), 974 pg/ml (± 182) and 2230 pg/ml (± 410), respectively, in tuberculosis patients receiving treatment for less than 1 month, those receiving treatment for more than 1 months and in healthy contacts. As is demonstrated in the histogram in figure 7, the majority of patients had still had relatively suppressed PPD stimulated IFN-γ responses at 2 months. However, a substantial number of patients had very active IFN-γ responses at 2 months, probably indicating good recovery of protective responses, in this group of patients.

The risk of a poor outcome (treatment failure, relapse or death) during the 24 months period was 9% higher for each 100 unit increase in the IFN-γ response to stimulation with the mitogen PHA. The importance of this finding is not clear, but it possibly suggests that those patients with increased non-specific immune activation were at greater risk of a poor treatment response, after adjustment for the other factors in the model.

Figure 7. The distribution of whole blood PPD-stimulated IFN-γ production in the study population after 2 months of treatment in hospital.



Higher creatinine concentrations were associated with better treatment outcomes by 24 months. A 10 µmol/l increase in creatinine was associated with a 40 % reduction in the risk of treatment failure, death or relapse by 24 months. Like urea levels, higher creatinine concentrations might indicate less wasting, better nutritional status and less severe disease; these factors may be associated with better immunity and, or socio-economic circumstances which, in turn may reduce the risk to patients of an unfavorable outcome.

DISCUSSION

The discordance between the different measures was surprising. The methods used for sputum microscopy and culture at 2 months, and record of treatment outcomes in the register, were those routinely used by the health care services in the region; they should be verified. The weight gain after admission to the hospital was likely to be confounded by altered availability of nutrients. Some patients received streptomycin during the intensive phase of treatment if the use of ethambutol was contraindicated, or if they were receiving retreatment regimens; and retreatment patients received ethambutol during the continuation phase. Treatment adherence was not accounted for after hospital discharge and may have confounded the measures of outcome after 2 months. The disposition of many patients 24 months after admission to the hospital was not known. In spite of these weaknesses, several important findings emerged from the evaluation treatment response and associated factors. Rifampicin, isoniazid and ethambutol concentrations and pyrazinamide dose/kg, respectively, associated with different effects on the treatment response measures. A protective type-1 immune response at 2 months was the most important predictor of the outcome at 24 months, and HIV-infection was associated with a worse prognosis.

The dose related reduction in weight gain associated with pyrazinamide supports contemporary dosing guidelines[128], which advocate lower doses than those prescribed for many of the patients in this study.

While higher ethambutol concentrations were associated with reduced weight gain, they were also associated with improved sputum sterilization at 2 months. The findings might suggest that target ethambutol concentrations lie somewhere within the ranges achieved in this patient cohort. But, arguably, it may be more important for patients to achieve 2 sterilization of the sputum at 2 months than to gain weight.

Both rifampicin and ethambutol levels were important determinants of the sputum results at the end of the intensive phase of treatment. Why rifampicin levels were more closely associated with the smear result yet ethambutol levels related to culture results is a question beyond the scope of this investigation, and is not resolved by the empirical findings reported here. Possibly, the different drug effects on the metabolically different sub-populations of *Mycobacterium tuberculosis* may account for the disparate findings.

The pharmacokinetic measure for each drug corresponding most closely to efficacy has not been determined for the antituberculosis agents. It has been suggested that peak concentrations are important for the antimicrobials acting on intracellular targets, whereas the time above the MIC might be the measure that most accurately predicts the action of drugs on cell wall components. However, it is simplistic to apply these principles to this setting where efficacy is measured by the presence and viability of organisms in the sputum. Furthermore, drug action may be complicated by factors such as postantibiotic effects, unbound drug penetration to, and residence time at different sites within the lung, the changing susceptibility of the organisms depending on their metabolic activity at the site of their occupation, etc. In this study AUC1 appears to be the most important measure of rifampicin for clearance of *Mycobacterium tuberculosis* from the sputum smear, while the peak concentration of ethambutol appears to be the measure most closely related to sputum sterilization.

While both sputum microscopy and culture are recognized as markers of long-term outcome[47,163,164], the positive predictive value of the early measures is low [47,137,162]. In this study the 2 month markers were discordant and only the smear result was associated with the 24 month prognosis.

Immune suppression early in treatment has been associated with better sterilizing activity of drug treatment[165,166]. This might, in part, explain the disparity between the 24 month outcome and the early culture result, and why drug factors were important for sputum sterilization at 2 months but not the 24 month prognosis; perhaps in those with better immunity, the treatment had reduced sterilizing activity, but their superior immunity may have protected against reactivation or reinfection after treatment completion. Protection against reinfection is of particular importance in high prevalence communities; in another Western Cape community, a high proportion of patients presenting with tuberculosis in the months following treatment in had infection with a new strain of *Mycobacterium tuberculsosis*[157].

The association of higher isoniazid levels with worse TB-register recorded outcomes is of concern and deserves further investigation.

Other studies have found gender to be a prognostic factor[43,45]. In this study, in spite of significantly lower rifampicin, isoniazid and pyrazinamide levels in men, sex was not a significant determinant of treatment response.

Some of the analyses were underpowered: in particular, the regression of highly variable continuous covariates (like the measures of rifampicin levels) and dichotomous covariates with only a few subjects in one group (e.g. HIV-infection) on the 2 month sputum culture (where only 12 subjects had positive cultures); and in the survival analysis (where there were many censored observations and the variability attributable to adherence was not accounted for). Furthermore, the patient group studied might have had inherent differences to the majority of patients who do not require hospitalization, and the results should not be generalized without due caution.

VI

CONCLUSIONS

As outlined below, most of the study objectives were met. The important findings are highlighted. The need to establish target drug concentration ranges and confirm the determinants of treatment response in adequately powered studies amongst more representative populations of tuberculosis patients is emphasized.

Objective i; The concentrations of rifampicin, isoniazid, pyrazinamide and ethambutol measured in 142 adult patients at Brewelskloof TB Hospital on the outskirts of Worcester, Western Cape. Relatively intensive sampling was used over an 8 hour time period following administration of the patients' routinely prescribed medicines. The pharmacokinetic profile for each drug was obtained in each patient on a single occasion after 2 months of antituberculosis therapy in the hospital.

Objective ii; From the pharmacokinetic profiles determined, the 5th to 95th centile ranges for the pharmacokinetic measures for each of the drugs in this patient population were determined. The study did not address the alterations in drug concentrations that might occur over time. For example, changes in rifampicin concentration due to autoinduction following repeated doses; and changes in protein binding with resolution of the inflammatory response leading to altered drug distribution. Neither did the study evaluate interoccasional variability in drug concentrations within a patient; thus, the accuracy with which drug measurement on a single occasion predicts the drug exposure on other occasions was not assessed. The study was not designed to determine the optimal drug concentrations for efficacy or toxicity. Since the ranges of drug concentrations found within in this cohort of patients affected the treatment response, it is suggested that concentrations are not optimal in all patients. Properly designed and adequately powered studies should be implemented to define the optimal ranges for the drug concentrations.

Objective iii; The drug level at a single time point 2 hours after drug dosing was highly correlated with isoniazid, pyrazinamide and rifampicin peak concentrations; the 2.5-hour and 3-hour time points, respectively, reflected the Cmax of rifampicin and ethambutol more accurately. The amounts of rifampicin measured in the urine (volume x rifampicin concentration) identified those patients with very low systemic rifampicin concentrations. But the urinary collections were less accurate than a single systemic plasma concentration for prediction of the peak concentration or AUC of rifampicin. The amounts of isoniazid, pyrazinamide and ethambutol excreted in the urine were not measured.

Objective iv: Several patient and formulation characteristics were identified as important determinants of the pharmacokinetics of rifampicin, isoniazid, pyrazinamide and ethambutol, respectively.

Formulation factors were confirmed to be important determinants of rifampicin bioavailability. The relative contributions of the raw material properties, the manufacturing processes and storage conditions to the variability in bioavailability are uncertain. Further studies are required to guide policy to ensure that the variations in bioavailability attributable to product quality are minimized.

The influence of HIV-infection in reducing rifampicin, pyrazinamide and ethambutol levels in a tuberculosis patient population was established. A survey in 2003 found that 55% of tuberculosis patients in South Africa are co-infected with HIV[168]. Therefore, significant reductions in the drug concentrations in this patient population may have important implications for tuberculosis control in the country. Further studies are required to establish the effect of HIV progression on the drug levels, and whether dosage adjustments should be implemented in certain categories of HIV-infected tuberculosis patients.

Other significant covariate effects included sex, smoking habits, and various markers of disease and nutrition. Clearly our understanding of the interaction mechanisms of genetic and physiological constitution, disease, nutrition, environment and pharmacokinetics are rudimentary. Expanding our knowledge of these may ultimately lead to more refined dosing strategies.

The considerable residual variability could be the result of various factors as discussed previously. P-glycoprotein expression and activity may be of particular importance for rifampicin concentration. Moreover, as the amount of rifampicin is a determinant of CYP3A4 expression[106]; rifampicin concentrations might be important for prediction of the severity of drug-drug interactions. The converging epidemics of tuberculosis and HIV dictate that large numbers of patients in Africa and elsewhere will simultaneously require antiretroviral and antituberculosis treatments. Rifampicin containing regimens offer the best available antituberculosis treatments for HIV-infected tuberculosis patients. Pharmacokinetic interactions between rifampicin and the antiretroviral non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) are described but have not been adequately evaluated to guide treatment regimen and dosing strategies[169,160]. As the NNRTIs and PIs are P-glycoprotein and cytochrome P450 enzyme substrates; not only is there a need to study the effect of the presence

of concomitant rifampicin per se on the antiretroviral drug concentrations in various HAART (highly active antiretroviral treatment) regimens prescribed with rifampicin in TB/HIV patients; but the impact of the wide variations in rifampicin levels seen in patients on the severity of the drug interactions deserves investigation.

Objective v: The response to treatment was measured in the cohort of patients by means of the proportional weight gain, the sputum smear conversion and the sputum sterilization (measured by Bactec culture) after 2 months of treatment in Brewelskloof Hospital; and also the TB register recorded outcome at the end of the treatment period; and the treatment failure, relapse and death rates up 24 months after the time of hospital admission. The weaknesses discussed previously should be addressed in more rigorously designed pharmacokinetic studies of drug efficacy and toxicity to verify the results and to establish target therapeutic drug concentrations. None-the-less, higher doses of pyrazinamide and higher ethambutol levels were associated with reduced weight gain, higher concentrations of rifampicin and ethambutol were associated with sputum conversion at 2 months, and higher concentrations of isoniazid were associated with unfavourable TB register outcomes. Furthermore, markers of immunity at 2 months were the predominant determinants of the prognosis at 24 months after admission.

Objective vi: As described above, low rifampicin and ethambutol concentrations were associated with an increased risk of the persistence (at 2 months) of *Mycobacterium tuberculosis* in sputum smears and cultures, respectively. Neither low levels of rifampicin, isoniazid, pyrazinamide, nor ethambutol were associated with an increased risk of a treatment failure, death or relapse during the 2 years following the time of admission to Brewelskloof Hospital. As outlined in the 'Methods' chapter, the study was underpowered to detect a significant differences in rifampicin levels between responders and nonresponders (based on sputum culture results at 2 months) without adjustment for other risk factors.

Objective vii: The cytokine markers of immunity were measured in most of the study population, and, as described above, had important bearing on the prognosis 2 years after the time of admission to the hospital.

Objective viii: The susceptibility of the infecting strains of *M. tuberculosis* in this population to isoniazid and rifampicin was measured in only a small proportion of study patients; thus preventing evaluation of the impact of the mini-MIC on treatment response. However, the narrow range of susceptibilities observed indicates that for

most patients with drug sensitive organisms, the mini-MIC is unlikely to contribute to differences in treatment response.

Finally the extent to which the results can be generalized is limited by the atypical nature of the study population. Patients admitted to hospital for treatment comprise a minority, and have inherent differences to those treated as outpatients.

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appendix 1.1

PROTOCOL

S-P2: PHARMACOKINETICS OF ANTITUBERCULOSIS DRUGS IN PATIENTS RESPONDING POORLY TO TREATMENT

Investigators: H McIlleron, P Wash, A Burger, J Wilkins, G Gabriels, J Cockcroft, P Smith, P Folb

LITERATURE REVIEW AND BROAD SCIENTIFIC GOALS:
The implementation of DOTS programmes internationally has lead to promising improvements in the control of tuberculosis; with many countries achieving high cure rates using this WHO TB control package (1, 2). Low success rates under DOTS programmes in Africa (average 58%; South Africa 69% in 1996) are largely due to failure of patients to complete treatment and incomplete evaluation of treatment outcomes (2). However there remains a small but significant group of patients who have a delayed or inadequate response to treatment under conditions of close supervision and support. These patients who do not respond to treatment as expected, and in whom one is assured that the *Mycobacterium tuberculosis* organism is susceptible to the drugs given, and the patient is taking the medication as prescribed, present a management dilemma.

Failure to achieve adequate drug serum concentrations is believed to be one of several reasons why 2 to 5% of compliant tuberculosis patients either fail drug therapy of relapse after therapy (24). Low serum levels of antituberculosis drugs in patients on standard doses have been reported in a number of studies (3,4,5). In some studies low levels of the antituberculosis agents are attributed to HIV infection (3,6). This relationship has not been supported by other studies; low drug levels could not be related to HIV infection, diarrhoea, or CD4 lymphocyte counts in tuberculosis patients presenting to a hospital in Nairobi (4). studies in tuberculosis patients at hospitals in Cape Town (7) and Durban (8) found no significant differences in drug levels between AIDS patients and HIV negative patients; and a study amongst Thai AIDS patients failed to demonstrate delayed or reduced absorption of rifampicin (32). A further study (5) showed a significant number of HIV negative patients who were responding poorly to therapy to have low 2 hour levels of rifampicin (64%) and isoniazid (68%).

Factors influencing levels of the antituberculosis drugs are poorly defined; apart from the controversial effect of HIV infection, factors that may be associated with low drug levels may include genetic differences in metabolic rates, environmental influences on metabolism (9), concomitant food (10,13), concomitant illness, concomitant medication (3,11,12) , nutritional status and diet (9,4). The influence of acetylator genotype on the elimination of isoniazid has been well defined (14), and is believed to be of importance for once weekly dosing regimes, but not to significantly influence the outcome of 5 or 3 times weekly dosing schedules(15,23). The drug exposure in patients given doses once weekly and who eliminate the drug rapidly, is not sufficient to retard growth of the organism for long enough and more regrowth occurs between doses. In slow acetylators, drug exposure is 3 times greater, and sufficient lag in the growth of the organisms is achieved for once weekly dosing to be effective(15). Combined with other factors which decrease levels of isoniazid, intermediate levels of organism resistance, or a compromised immune system the acetylator status may be an important factor in thrice weekly and daily dosing regimens.

The use of once weekly isoniazid combined with rifapentine in the continuation phase of treatment in HIV-seropositive patients lead to the emergence of an alarmingly high proportion of acquired rifamycin monoresistance in these patients; a finding not seen in HIV-seronegative patients (23). It is hypothesised that the emergence of rifamycin resistance was a result of periodic functional monotherapy in these patients; a situation which would theoretically be aggravated by a rapid acetylation phenotype or other factors which tend to decrease isoniazid levels. The same study demonstrated a relatively high relapse rate in HIV-seropositive patients, whether they received once weekly rifapentine plus isoniazid or standard therapy of twice weekly rifampicin plus isoniazid (23). This finding demonstrates the importance of host immunity, and combined with the possibility that certain HIV-seropositive

The development of an MRC site in Durban by Dr Colin Pillai to conduct similar bioavailability studies in conjunction with the WHO has added data from a different population. The data accumulated thus far can be used to develop models of the population kinetics for the drugs. However this data has limitations for the development of guidelines for therapeutic drug monitoring. Most of the data has been generated from studies involving healthy volunteers who have different pharmacokinetic profiles to patients, in particular their CYP-450 enzyme systems are not induced by exposure to rifampicin. In addition the healthy volunteer population has been of well nourished, non-smoking individuals who have minimal exposure to alcohol and concomitant drugs

The department was approached by clinicians at the Brewelskloof Hospital in Worcester to measure drug concentrations in the blood of patients who respond poorly to treatment, yet are known to have sensitive organisms and under hospital supervised DOT.

ARISING OBJECTIVES.

This project aims to help to resolve some of the dilemmas faced by clinicians treating tuberculosis patients who are responding poorly to treatment. Factors associated with a poor outcome will be sought. The value and use of Therapeutic Drug Monitoring of tuberculosis patients will be explored in this context.

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patients may achieve inadequate serum concentrations of the drugs on standard drug doses, supports the use of therapeutic drug monitoring in these patients.

The detection of low drug levels may be even more important for rifampicin. An early study demonstrated rifampicin doses of less than 9mg/kg may be inadequate for the treatment of pulmonary tuberculosis (25). Early bactericidal activity studies support the dose-dependence of rifampicin's antibactericidal activity (28,29,30). Coupled with a smaller Cmax : MIC ratio for rifampicin than isoniazid and greater protein binding, lower than normal concentrations of rifampicin may be more critical. In addition, the rifamycins are less reliably absorbed than isoniazid (23).
Consistent with theory that drugs acting on intra-microbial targets generally exhibit concentration-dependent killing and require high plasma concentrations in relation to MIC for optimal antimicrobial effects (31)

Therapeutic drug monitoring is a tool that could be used to identify patients who require increased doses of the antituberculosis drugs and to determine doses which would bring the drug levels within the normal ranges (16,17,18,27). Patients at risk of low drug levels should ideally be identified as early as possible to prevent treatment failure or the development of resistance.

Data from bioavailability studies in both patients and healthy volunteers (in the Department of Pharmacology at the University of Cape Town) suggests wide inter-individual variability of the maximal concentrations (Cmax) and area under the concentration-time curve (AUC) for rifampicin and isoniazid.

There is little published about the inter-occasional variability of the pharmacokinetics of anti-tuberculosis drugs within the same individual.

Intra- and inter-individual variations have important consequences for the technique of therapeutic drug monitoring. Some studies have used a single blood sample taken 2 hours after drug administration to detect low drug levels (3,5). Another study (7) found that the 2 hour level alone did not adequately reflect the Cmax derived form a more extensive sampling schedule. Further study could allow the development of guidelines on blood and / or urine sampling schedules that could identify patients with low drug levels in the most cost effective manner.

A better understanding of the relationship between drug concentrations, organism sensitivity, immunity and the response to drug therapy could facilitate appropriate treatment for those patients who fail to respond as expected to standard treatment regimens.

WORK WHICH HAS LED UP TO THE PROJECT:
A study on the effect of concomitant food intake on the bioavailability of rifampicin, isoniazid and pyrazinamide (10) initiated an interest in the pharmacokinetics of the anti-tuberculosis drugs in the Department of Pharmacology at the University of Cape Town. Subsequently a further study of the kinetics of rifampicin, isoniazid and pyrazinamide in AIDS patients and HIV negative patients (7) provided further opportunity for the development of validated assays for these drugs. Since 1996 twenty single dose cross-over studies to assess the bioavailability of new formulations of anti-tuberculosis drugs have been conducted by the department in collaboration with the National Tuberculosis Research Programme of the South African Medical Research Council (MRC) under Dr Bernard Fourie and the World Health Organisation (WHO). This work has led to the accumulation of a considerable amount of data on the pharmacokinetics of rifampicin, isoniazid, pyrazinamide and ethambutol in both healthy volunteers and patients. In addition considerable expertise has been achieved in the laboratory, enabling the reliable and consistent processing of large quantities of blood and urine samples according to validated HPLC methods. The infrastructure and expertise of the laboratory already established form a strong background on which to build further pharmacokinetic studies.

Study site: Brewelskloof Hospital in Worcester is designated primarily for the in-patient care of tuberculosis patients. Patients are referred to Brewelskloof from tuberculosis clinics throughout the Western Cape, and occasionally further afield. Reasons for referral include suspected non-compliance, poor response to treatment, poor socioeconomic circumstances and drug resistance. It is a 270-bed hospital with 4 medical doctors and competent nursing personnel. X-ray and bronchoscopy facilities are available. Sputum samples are sent to SAIMR in Greenpoint, Cape Town, for microscopy, culture and sensitivity testing.

Study population: Eligible in-patients receiving at least 8 weeks but not more than 9 weeks of daily antituberculosis therapy with first line agents according to regimens for new or retreatment patients in Brewelskloof Hospital. Approximately 200 patients will be recruited.

Inclusion criteria:

- Informed consent
- ≥ 18 years old
- Pulmonary tuberculosis: diagnosed by positive sputum or sputum culture
- Positive sputum (direct or culture) on admission to BKH
- New or retreatment cases of tuberculosis on treatment with isoniazid, rifampicin, pyrazinamide with or without ethambutol and / or an aminoglycoside

Exclusion criteria:

- critically ill
- Contraindication to multiple blood sampling eg mental confusion / poor venous access
- Known resistance to isoniazid or rifampicin before study initiation

Sample size. A sample size of 200 at Brewelskloof Hospital is estimated to represent approximately 20 (10%) patients in whom the response would be regarded as poor by the clinicians caring for them. A sample size of 20 in this group is estimated to be required to detect a significant difference between those who respond well and this group, with 80% power and a 5% chance of incorrectly rejecting the null hypothesis.

Procedure:

Admission data: On admission all potentially eligible patients will have baseline data collected and recorded. These include:

- symptoms of cough, dyspnoea, night sweats, chest pain and appetite
- weight
- chest x-ray
- direct sputum microscopy for acid fast bacilli
- sputum culture for Mycobacterium tuberculosis, and susceptibility to isoniazid and rifampicin.

Assessment after 2 months of therapy: Eligible patients will be identified between 8 and 9 weeks after admission for DOT and written informed consent obtained before the following parameters will be documented (at 8 to 9 weeks)

- age
- gender
- race
- place of birth
- current place of residence

- past medical history
- tuberculosis treatment history
- concomitant illness or diseases
- current tuberculosis treatment including drug doses and dosing interval
- concomitant medication or treatment
- current symptoms of cough, chest pain, night sweats and appetite

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PROTOCOL

OBJECTIVES:

1. To quantify the number of patients in the study population responding poorly to 1st line antituberculosis therapy.
2. To determine the relationship between response to treatment and drug levels.
3. To evaluate the association between the degree of in vitro sensitivity of the pathogen to rifampicin and isoniazid, and the response to therapy.
4. To evaluate the association between the type of immune response, and the response to therapy.
5. To explore associations between drug levels and age, gender, race, acetylator status, extent of disease, smoking, the use of concomitant drugs, alcohol, cannabis, mandrax, HIV infection and chronic diarrhoea.
6. To determine efficient methods of blood and / or urine sampling for the identification of patients with low drug levels.
7. To evaluate inter-occasional differences in tuberculosis drug levels.

METHODS:

Study design. A prospective cohort pharmacokinetic study

6, 9, 12, 18 and 24 months. If the culture is positive, the susceptibility of the organism to rifampicin and isoniazid will be determined as described below (analytical methods). Additionally, sputum samples will be collected for microscopy and culture should the patient deteriorate or present with signs or symptoms of worsening or recurrence of tuberculosis, and 2 sputum samples will be collected for evaluation by direct microscopy for acid fast bacilli in accordance with current clinic practice.

Study modifications: The single drug formulations routinely used in the hospital will be administered in the doses routinely prescribed for each patient – the doses in the table below are used by clinicians as a guideline.

Wt	*<33kg*	*33 – 50kg*	*>50kg*
INH	200 mg/dose	300 mg/dose	300 mg/dose
Rifampicin	300 mg/dose	450 mg/dose	600 mg/dose
Pyrazinamide	1000 mg/dose	1500 mg/dose	2000 mg/do se
Ethambutol	800 mg/dose	1000 mg/dose	1200 mg/dose

The batch number and expiry dates of all drugs used will be recorded and the quality of each batch will be tested by in vitro methods prior to use.

Subject consent: The study purpose and procedure will be explained and will include explanation of risks and that participation will not offer any therapeutic benefit to the patient. Participation in the study will be voluntary and should a subject wish to withdraw from the study at any stage they will do so without prejudice to him or her for doing so. Written informed consent in Afrikaans, English or Xhosa will be obtained from all patients who volunteer to participate in the study prior to enrolment. Consent will be obtained for HIV testing. Subjects will receive pre- and post-test counseling.

Ethical considerations: The protocol must be approved by the Research Ethics Committee of the University of Cape Town's Medical Faculty and by independent institutional ethical review of a committee convened for this purpose at Brewelskloof Hospital. The study will be conducted in accordance with the moral, ethical and scientific principles governing clinical research as set out in the Declaration of Helsinki.

Insurance and liability: The University of Cape Town subscribes to an insurance policy for the protection of staff and students, engaged in research, against liability claims. Students are insured (under the Student Personal Accident insurance policy) to the value of R5000 for accidental injuries incurred as a result of UCT related activities or duties. Staff are eligible to claim compensation under the Workmen's Compensation Act for injuries or disease, incurred in the course of undertaking their employment.

ANALYTICAL METHODS:

Drug assay methodology: Rifampicin, isoniazid, pyrazinamide and ethambutol levels will be determined in the Department of Pharmacology, University of Cape Town according to validated HPLC methods (21,22).

Acetylator status determination: Acetylator status will be assessed and determined by the elimination half-life of isoniazid, the ratio of N-acetylisoniazid to isoniazid over various time points subsequent to dosing. Statistical analysis will be implemented in developing the criteria for each acetylator class.

Sputum microscopy, culture and drug susceptibility cultures to isoniazid and rifampicin: These will be conducted by validated methods in conjunction with the SAIMR at Green Point and the Department of Medical Microbiology at Groote Schuur Hospital.

Routinely, drug susceptibility testing will be done for rifampicin and isoniazid on culture positive specimens. The Bactec system will be used to determine susceptibility at 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/l for isoniazid and at 0.06, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/l for rifampicin.

- weight
- height
- clinical examination

- chest x-ray
- full blood count and differential
- erythrocyte sedimentation rate
- urea, creatinine, ALT, AST, GGT, AP, TB, albumin, total protein
- HIV
- CD3, CD4 and CD8 counts; plasma cytokine levels and whole blood cytokine production
- acetylator genotype
- sputum direct microscopy for AFBs, culture for TB and sensitivity to rifampicin and isoniazid, if still positive

Pharmacokinetic assessment: After 8 to 9 weeks of treatment the pharmacokinetic profiles for rifampicin, isoniazid, pyrazinamide and ethambutol will be determined according to the following procedure:

- After an overnight fast rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin will be administered simultaneously with 200ml of water according to the standard doses for the patient concerned. The drug doses administered will not be the altered for the study. Drug ingestion will be observed by an investigator or investigator's assistant and the exact time of drug administration will be recorded.

- Blood samples for the determination of plasma levels of rifampicin, isoniazid, pyrazinamide and ethambutol will be drawn at the following times: 1) within 45 minutes before drug administration
    2) 0.5 hours after drug administration
    3) 1.0 hours after drug administration
    4) 1.5 hours after drug administration
    5) 2.0 hours after drug administration
    6) 2.5 hours after drug administration
    7) 3.0 hours after drug administration
    8) 4.0 hours after drug administration
    9) 6.0 hours after drug administration
    10) 8.0 hours after drug administration

- Complete urine collections will be made from 2 to 4 hours, 4 to 6 hours and 6 to 8 hours after drug administration. The volumes and exact times of the collections will be recorded.

- Subjects will remain semi-upright for 2 hours after drug administration and no food or fluid will be allowed during this period

- The timing and content of meals, snacks and fluids taken during the observation period will be recorded.

Inter-occasional variability: In a subset of 60 patients, the pharmacokinetic profiles of the drugs will sampled on 3 occasions one week apart, in order to assess interoccasional variability in the drug levels within patients.

Evaluation of response to therapy: 'Early' response to therapy (after 2 months in-patient directly observed therapy) will be graded according to a scoring system using sputum microscopy and culture, chest x-ray, weight and symptoms. The parameters will be recorded both at admission and at 8 to 9 weeks (see appendix 2 – TB progress rating at 2 months).

Assessment of therapeutic progress and relapse detection ('Late' response to treatment): A sputum sample will be examined bacteriologically (by direct smear and culture) between 7 and 9 weeks after admission for treatment in Brewelskloof Hospital (as stated above) and at

Chemical pathology, haematology and immunology evaluations: The haematology, biochemistry, serology and immunology service laboratories of the University of Cape Town will analyze the relevant blood tests taken at 8 to 9 weeks.

Statistical analysis: Plasma concentration profiles will be derived for each patient for rifampicin, isoniazid, pyrazinamide and ethambutol. The pharmacokinetic parameters of importance for bioavailability will be calculated using the WinNonLin software package. The relationship between the response to therapy, pharmacokinetic parameters, immune status markers, and organism sensitivity to rifampicin and isoniazid will be described. The effect of age, gender, race, acetylator status, extent of disease, smoking, use of concomitant drugs, alcohol, cannabis, mandrax, HIV infection and chronic diarrhoea on the pharmacokinetic profiles will be assessed using NONMEM modeling systems. Inter-occasional differences in the pharmacokinetics of the drugs will be described. The relationship between low drug concentrations, and susceptibility of the organism to isoniazid and rifampicin, at 8 to 9 weeks after admission, and treatment outcome will be assessed.

I understand that the study is to improve the understanding of how drug levels, tuberculosis sensitivity to drug levels and other individual patient factors influence the effectiveness of TB treatment.

I understand that the study involves the documentation of personal and treatment details, a physical examination, collection of blood samples (about 25ml of blood), a chest x-ray and sputum collection. Laboratory evaluation of the blood will include testing for the HIV virus, haematological, biochemical and immunological status.

In addition, on one or three occasions: I will fast overnight before serial blood samples totalling about 110ml will be taken over a period of eight hours after the swallowing of my medication the next day. A cannula will be inserted into an arm vein for the collection of these blood samples. Three, 2hour urine collections will also be collected during the 8 hour period(s).

I understand that the study also involves the collection of a sputum samples at 2 months and at 6, 9, 12, 18 and 24 months after the initiation of treatment at Brewelskloof Hospital. I understand that I am obliged to present at the clinic to which I am referred at the required times.

I understand that participation in the study does not hold any treatment benefit.

I have been given the opportunity to ask any questions concerning the study and am satisfied with the explanation or advice given.

I have truthfully answered all the questions necessary to complete the data collection forms.

I agree to comply with the instructions given to me by the investigators or their assistants.

I understand that the information collected will be treated confidentially, and that only the investigators and their assistants will be able to link any of the information collected to my name.

I understand that I am free to withdraw from the study at any time without prejudice to me for doing so.

I am aware that this study has been subjected to review by an ethics committee.

NAME OF SUBJECT	SIGNATURE	DATE
NAME OF INVESTIGATOR / INVESTIGATOR'S ASSISTANT	SIGNATURE	DATE

appendix 1.2

CONSENT FORM

Subject no.

CONSENT TO PARTICIPATE IN A CLINICAL STUDY
PHARMACOKINETICS OF ANTITUBERCULOSIS DRUGS IN PATIENTS RESPONDING POORLY TO THERAPY.

appendix 1.3

CASE RECORD FORM

DATA COLLECTION FORM

Subject no: _____ **Date:** _____

Ward: _____ **Investigator:** _____

Folder number: _____ **Date of admission:** _____

Name: _____

Age: _____ **Sex:** male / female

Race: A | B | C | W | Other

Home address: _____

Place of birth: _____

ELIGIBILITY:

☐ *Pulmonary tuberculosis confirmed by positive sputum for M. tuberculosis: direct microscopy or culture*

☐ *Informed consent*

☐ *consent to HIV testing*

☐ *≥ 16 years*

☐ *On daily inpatient treatment with rifampicin, isoniazid, pyrazinamide, +/- ethambutol, +/- an aminoglycoside for 8 weeks at Brewelskloof Hospital.*

☐ *not critically ill and no contraindications to multiple blood sampling*

☐ *no known resistance to isoniazid or rifampicin*

New TB patient		Retreatment patient	

Previous TB: history and treatment RI: treatment interruptions RF: treatment failures RR: treatment relapses E: episodes	*Date:*	RI	RF	RR	E

Past **medical history**:	

Current / chronic disease:	

Concomitant medication:	

Drug allergies/ reactions:	

Alcohol intake:	*In year before admission:* *Since admission:*

Smoking:	*In year before admission:* *Since admission:*

Cannabis:	*In year before admission:* *Since admission:*

Other recreational **drugs**:	*In year before admission:* *Since admission:*

CURRENT TB	*Date of onset:*
Diagnosis:	*Date:* *Sputum – direct microscopy:* *Sputum – culture:*
Treatment prior to admission:	*Date of initiation of treatment:* *Drugs and dosing – H:* *R:* *Z:* *E:* *S:*

Current episode of TB cont...

Symptoms on ***admission****:*	*Cough:* *Dyspnoea:* *Chest pain:* *Night sweats:* *Appetite:*
Examination on ***admission****:*	
Weight:	kg
CXR:	*Date:* _____ *number:* _____ *Comment:*
Sputum:	*Date:* _____ *Direct microscopy:* _____ *culture:* _____ *(-/scant/+/++/+++)* *(-/days to + culture)*

Symptoms at ***2/12****:*	*Cough:* *Dyspnoea:* *Chest pain:* *Night sweats:* *Appetite:*
Examination at ***2/12****:*	
Weight:	kg
Height:	cm
CXR:	*Date:* _____ *number:* _____ *Comment:*
Sputum:	*Date:* _____ *Direct microscopy:* _____ *culture:* _____ *(-/scant/+/++/+++)* *(-/days to + culture)*
Treatment during admission:	*H:* _____ *mg* *details :- document date treatment started if other than admission date, altered dosage regimens, missed doses, etc.* *R:* _____ *mg* *E:* _____ *mg* *Z:* _____ *mg* *S:* _____ *mg* *Other:*

Discharge date:	
Clinic details:	

RESULTS

'Early' response to treatment
Response rating at 2/12: /20

'Late' response to treatment																	
Sputum:	*2m*	*3m*	*4m*	*5m*	*6m*	*7m*	*8m*										
Date:																	
Direct microscopy.																	
Culture:																	

IMMUNOLOGY	
Date:	*Immunophenotyping:*
	Plasma cytokine levels:
	Stimulated whole blood cytokine production:

MICRO - 'mini-MICs'	
Date:	*Isoniazid:*
	Rifampicin:

Date:	*Haematology:*
	Chemical pathology:
	Virology:

Subject no. _____

PK SAMPLING / ADVERSE EVENTS FORM

DATE of PK sampling: / /

Drug administration:

Drug	Dose	Exact administration time	Formulation / brand	Batch no.	Exp. date
Rifampicin					
Isoniazid					
Pyrazinamide					
Ethambutol					
Notes:					

Blood sampling times:

Sample	Hrs after drug administration	Exact time of sampling (hh:mm)
0	**Pre-drug sample**	
1	**0.5**	
2	**1.0**	
3	**1.5**	
4	**2.0**	
5	**2.5**	
6	**3.0**	
7	**4.0**	
8	**6.0**	
9	**8.0**	

Urine sampling times / volume:

Sample	Time after drug administration	Exact collection time	Volume of collection (ml)
Pre-collection void	**2 hrs**		
1	**2-4 hrs**		
2	**4-6 hrs**		
3	**6-8 hrs**		

appendix 1.4

LETTERS OF ETHICAL APPROVAL

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

14 May 1999

REC REF : # 122/99

Dr H McIlleron
Pharmacology

Dear Dr McIlleron

THERAPEUTIC DRUG MONITORING IN TUBERCULOSIS PATIENTS RESPONDING POORLY TO TREATMENT

I have pleasure in informing you that the above study has been **formally approved** by the Research Ethics Committee on 14 May 1999.

Included is a list of Research Ethics Committee Members who have formally approved your protocol.

Please quote the above Reference number in all correspondence.

Yours sincerely,

F.J. Folb 15/5/1999

Approval only by person not present for REC, in interest of myself. FJ.

PROFESSOR FOLB
CHAIR: RESEARCH ETHICS COMMITTEE

Queries: Martha Jacobs
Research Ethics Committee
Room 212 Werner and Beit
UCT Medical School
Anzio Road, Observatory, 7925
Tel: (021) 406-6492 Fax: (021) 406-6390
E-Mail: martha@medicine.uct.ac.za

UNIVERSITY OF CAPE TOWN
RESEARCH ETHICS COMMITTEE 1998/9

Faculty of Medicine
Anzio Road, Observatory, 7925

1.	CHAIR:	**Professor P I Folb (Male)** MBChB, MD, FRCP, FCP (SA) Professor of Pharmacology; Director: World Health Organization Collaborating Centre for Drug Policy
2.	EX-OFFICIO:	**Professor JP de V van Niekerk (Male)** MBCHB, MD, MMed (RadD),DIH (RCP&S) Dean of Faculty of Medicine
3.	EX-OFFICIO:	**Professor A A Forder (Male)** MBChB, Mmed (Microbiology) Pathology Professor Medical Microbiology & Deputy Dean of Medicine
4.	EX-OFFICIO:	**Dr P Mitchell (Male)** MBChB, FCP (SA) Chief Medical Superintendent
5.	MEMBER:	**Professor D Beatty (Male)** MBChB, MD, FCP (Paed) (SA) Professor and Head of Department of Paediatrics & Child Health
6.	MEMBER:	**Professor SR Benatar (Male)** MBChB, FFA (SA), MRCP (UK), FRCP (London) FACP (Hons) Professor and Head of Department of Medicine, Director of UCT Bioethics Centre
7.	MEMBER:	**Professor DM Dent (Male)** MBChB, CHM, FRCS (UK), FCS (SA) Professor of Surgery
8.	MEMBER:	**Dr L Henley (Female)** PhD Lecturer/ Social Scientist in Department of Paediatrics & Child Health
9.	MEMBER:	**Professor R Kirsch (Male)** MBChB, MD, Dsc (Med), FCP (SA) Professor of Medicine, Executive Director of MRC/UCT Liver Research Centre
10.	MEMBER:	**Sr L Mtwisha (Female)** BA, Diploma in Nursing; Theatre Technique Matron – Groote Schuur Hospital (Theatre) (Female)

11. MEMBER: **Dr AH Robins (Male)**
MBChB, DPM, MD, MRC (Psych)
Senior Lecturer in Department of Pharmacology (Male)

12. MEMBER: **Ms V Stacey (Female)**
Anglican Chaplain - GSH

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

28 July 1999

ERC REF: # 122/99

Dr H Mclleron
Pharmacology

Dear Dr Mclleron

THERAPEUTIC DRUG MONITORING IN TUBERCULOSIS PATIENTS RESPONDING POORLY TO TREATMENT: DETAILED PROPOSAL

Thank you for your letter to the Research Ethics Committee dated 20 July 1999.

The contents have been noted and **approved**

Yours sincerely

P.D. Folb

PROFESSOR P FOLB
CHAIR: RESEARCH ETHICS COMMITTEE

Queries: Martha Jacobs
Research Ethics Committee
Room 212 Werner and Beit
UCT Medical School
Anzio Road, Observatory, 7925

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

17 April 2000

ERC REF NO. 122/99

Dr H McIlleron
Pharmacology

Dear Dr McIlleron

THERAPEUTIC DRUG MONITORING IN TUBERCULOSIS PATIENTS RESPONDING POORLY TO TREATMENT: AMENDMENTS 1 & 2

Thank you for your letter to the Research Ethics Committee, dated 15 March 2000.

Amendment 1 and Amendment 2 have been approved.

Please quote above Reference number in all correspondence.

Yours sincerely

PROFESSOR DM DENT
ACTING CHAIRPERSON

Queries: Martha Jacobs
Research Ethics Committee
Room 212 Werner and Beit Building
UCT Medical School
Anzio Road, Observatory, 7925
E-mail. Martha @ curie.uct.ac.za

FAX : FAKS

PROVINCIAL ADMINISTRATION : WESTERN CAPE
DEPARTMENT OF HEALTH

PROVINSIALE ADMINISTRASIE : WES-KAAP
DEPARTEMENT VAN GESONDHEID

INQOLA YENTSHONA-KAPA
ISEBE LEZEMPILO

OFFICE USE KANTOORGEBRUIK
Date / Datum : 19.11.2002

Ref No/Verw Nr. 19/5/1

To/Aan : The nurse in charge – Compressionsiae sterile

Fax/Faks No :

Boland District Municipality	023 347 3668
Witzenberg Local Municipality	023 316 1877
Breede Valley Local Municipality	023 347 3671
Breede River Local Municipality	023 347 3668
[illegible] District Municipality	028 425 1014
[illegible] Local Municipality	028 840 1114
Overberg Local Municipality	028 312 1893

Number of pages (including this one)/Aantal bladsye (voorblad ingesluit) : 3

Ref: Access to TB Registers

In the period between July 1999 and February 2001 n = 160 patients at Brewelskloof Hospital were part of a study measuring [illegible] isoniazid, pyrazinamide and ethambutol conducted by UCT Pharmacology Department.

Please be informed that permission has been granted for the study department to access the necessary TB registers at the various clinics throughout the region.

The study will assess the treatment outcomes up until two years after admission to Brewelskloof Hospital.

Attached please find the list of clinics to be visited. Dr H. Mordieron will be in contact with the persons in charge to make necessary arrangements.

Your co-operation in the above matter is greatly appreciated.

Thank you.

Yours faithfully / Die uwe
DIRECTOR: BOLAND/OVERBERG REGION

Enquiries/Navrae : Mrs. M. Johnson

Tel No : (023) 348 1434

PROVINCIAL ADMINISTRATION: WESTERN CAPE
DEPARTMENT OF HEALTH

PROVINSIALE ADMINISTRASIE: WES-KAAP
DEPARTEMENT VAN GESONDHEID

INQOLA YENTSHONA-KAPA
ISEBE LEZEMPILO

DIRECTOR/DIREKTEUR
BOLAND/OVERBERG REGION
PRIVATE BAG X3016
WORCESTER
6849

BOLAND/OVERBERGSTREEK
PRIVAATSAK X3016
WORCESTER
6849

TEL NO (023) 348 1400

FAX/FAKS (023) 3425841

appendix 2

SAMPLE SIZE ESTIMATION

SAMPLE SIZE CALCULATIONS:

22 patients with uncomplicated pulmonary tuberculosis participated in a bioequivalence study at DP Marais Hospital in Westlake, Cape Town in 1997. The Cmax and AUCt values used in this calculation were determined after doing they received a 600 mg dose of the reference product for rifampicin (Rimactane (Ciba) 150 mg capsules). Stata 7.0 (StataCorp, 2001) is used for the calculations.

Summary of Cmax and AUCt values:

Variable	*Obs*	*Mean*	*Std. Dev.*	*Min*	*Max*
Cmax	22	8.692932	3.45659	2.0867	15.0411
AUCt	22	34.708	12.91976	6.4872	54.1741

The proportion of patients at Brewelskloof Hospital with a sputum positive for *M. tuberculosis* after 2 months of treatment in hospital was estimated to be approximately 10%.

To detect a Cmax value in 10% of patients that is 25% less than that of the rest of the study population, with a power of 0.8 and alpha of 0.5. 18 patients would be required in the group responding poorly to treatment and 173 would be required in the group responding.

. sampsi 8.69 6.52, p(0.8) r(0.1) sd1(3.46) sd2(3.46) onesided
Estimated sample size for two-sample comparison of means

Test Ho: m1 = m2, where m1 is the mean in population 1 and m2 is the mean in population 2

Assumptions:
alpha = 0.0500 (one-sided)
power = 0.8000
m1 = 8.69
m2 = 6.52
sd1 = 3.46
sd2 = 3.46
n2/n1 = 0.10

Estimated required sample sizes:
n1 = 173
n2 = 18

To detect an AUCt value in 10% of patients that is 25% less than that of the rest of the study population, with a power of 0.8 and alpha of 0.5. 16patients would be required in the group responding poorly to treatment and 151 would be required in the group responding.

. sampsi 34.71 26.03, p(0.8) r(0.1) sd1(12.92) sd2(12.92) onesided

Estimated sample size for two-sample comparison of means

Test Ho: m1 = m2, where m1 is the mean in population 1 and m2 is the mean in population 2

Assumptions:
alpha = 0.0500 (one-sided)
power = 0.8000
m1 = 34.71
m2 = 26.03
sd1 = 12.92
sd2 = 12.92
n2/n1 = 0.10

Estimated required sample sizes:
n1 = 151
n2 = 16

appendix 3.1

DETERMINATION OF PLASMA RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE CONCENTRATIONS

1. **Title:** **A High Performance Liquid Chromatographic (HPLC) Method for the Simultaneous Determination of Isoniazid, Pyrazinamide and Rifampicin in Human Plasma**

2. **Scope:** This method applies to the assay used for the analysis of isoniazid, pyrazinamide and/or rifampicin in human blood samples collected during a clinical study, or therapeutic monitoring of patient samples.

3. **Purpose:** To describe a High Performance Liquid Chromatographic (HPLC) method for the simultaneous determination of isoniazid, pyrazinamide and rifampicin in human plasma performed at the Division of Pharmacology, Department of Medicine, University of Cape Town.

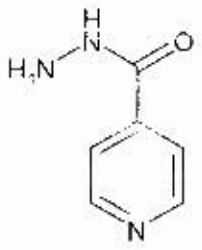
4. **General:** This assay is a simultaneous extraction of isoniazid, pyrazinamide and rifampicin, but only a simultaneous resolution of isoniazid and pyrazinamide. Different resolution conditions apply to rifampicin.

5. **Materials:**

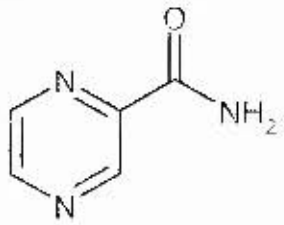
5.1. Description of assay components

Matrix	human plasma (heparin added as an anticoagulant)	
Sample Volume	500 µl	
Extraction method	Solid Phase Extraction	
Concentration ranges	Isoniazid:	0.2 - 15 µg/ml
	Pyrazinamide:	0.2 - 70 µg/ml
	Rifampicin:	0.3 – 25 µg/ml
Detection mode	UV	
Detection wavelength	270 nm	
Quantitation by	Peak Area	
Regression	Linear	
MW of analytes:	Isoniazid:	137.10 g/mol
	Pyrazinamide:	123.10 g/mol
	Rifampicin:	823.00 g/mol

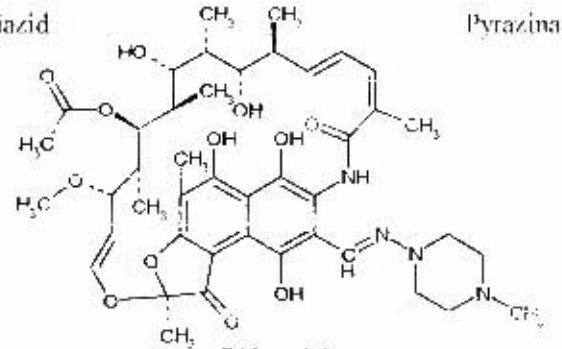
5.2 *Chemical Structures*



Isoniazid



Pyrazinamide



Rifampicin

5.3 *Instrumentation Specifications*

5.3.a. Common Extraction Equipment:

Hardware	Model/Brand	Supplier
Solid Phase Extraction Columns	Bond Elut C18, 3.0 ml	Varian/ Anatech
Vacuum Manifold and Pump	-	Analytichem Int.

5.3.b. Isoniazid and Pyrazinamide HPLC Equipment

Hardware	Model/Brand	Supplier
Solvent Pump	SP 8800	Spectra Physics
Autosampler	SP 8780 XR	Spectra Physics
Detector	UVIS	Linear
Integrator	SP 4290	Spectra Physics
Analytical Column (Ref No. 011230249153012)	Spherisorb S5 C8 25 cm x 0.46 cm	Waters
Guard Column: Upchurch C8 rev. phase 2.5 cm x 0.46 cm	Packed with Pelliguard LC 8	Anatech Packed in house

5.3.c. Rifampicin HPLC Equipment

Hardware	Model/Brand	Supplier
Solvent Pump	SP 8800	Spectra Physics
Autosampler	SP 8780	Spectra Physics
Detector	SP 8450	Spectra Physics
Integrator	SP 8780	Spectra Physics
Analytical Column (Ref No. 1204012)	Spherigrom Octyl 5µm C8; 4 mm x 150 mm	Anatech
Guard Column. Upchurch C8 rev. phase 2.5 cm x 0.46 cm	Packed with Pelliguard LC 8	Anatech Packed in house

5.4 *Chemical Specifications*

Chemical	Specifications	Supplier	Catalogue Number
Water	Type 1; Reagent grade	Millipore	In house purification
Methanol	>99.9%	BDH	15250
Acetonitrile	99.9%	BDH	15251
Trifluoroacetic Acid	99.0%	Riedel-de Haen	61030
Potassium Dihydrogen Phosphate	98.0%	Merck	4873
Isoniazid Reference Standard Isonicotinic Acid Hydrazide Lot No. 036H1179	>99.5% (Certificate of analysis attached)	Sigma	I 3377
Pyrazinamide Reference Standard Pyrazinoic Acid Amide Lot No. 109H1245	>99 % (Certificate of Analysis attached)	Sigma	P 7136
Rifampicin Crystalline Lot No. 078H0773	97.7% (Certificate of Analysis attached)	Sigma	R 3501

3.3.3. Precision, Accuracy, Stability and Recovery:

Table 2a. Validation data for rifampicin

	High QC *10 µg/ml*	*Medium QC* *5 µg/ml*	*Low QC* *2.5 µg/ml*
Average peak area	107400	59250	26400
%CV	4.38	6.04	6.08
Average % accuracy	104.6	112.4	108.1
% Recovery	105.4	119.1	119.7
n	14	15	15

Table 2b. Validation data for isoniazid

	High QC *10 µg/ml*	*Medium QC* *5 µg/ml*	*Low QC* *2.5 µg/ml*
Average peak area	97000	47600	25780
%CV	6.47	7.33	5.52
Average % accuracy	106.8	105.2	106.8
% Recovery	70.15	69.62	76.05
n	14	14	14

Table 2c. Validation data for pyrazinamide

	High QC *60 µg/ml*	*Medium QC* *30 µg/ml*	*Low QC* *15 µg/ml*
Average peak area	1.1016e^6	570100	268800
%CV	5.69	3.65	9.95
Average % accuracy	107.3	102.8	94.90
% Recovery	91.24	102.6	92.49
n	Accuracy: 9 Precision: 14	Accuracy: 6 Precision: 13	Accuracy: 6 Precision: 15

Figure 2a. Low Quality Control for 2.5 µg/ml rifampicin (5.37 min).

6. Methods:

6.1. Preparation of Stock Solutions

6.1.1 Preparation of a Secondary Reference Standard:

Analyte	Primary Storage Temp	Mass	Solvent	Volume of solvent	Final Concentration	Secondary Storage Temp
INH	Room Temp	10 mg	Water	10 ml	1 mg/ml	-20°C
PZA	Room Temp	40 mg	Methanol	10 ml	4 mg/ml	-80 °C
RIF	-20°C desiccated	10 mg	Methanol	10 ml	1 mg/ml	80 °C

Reputable chemical companies supply all reference standards (e.g. Sigma, Merck). A certificate of analysis accompanies all reference standards; either supplied by the company, or downloaded from the Internet. Records of the Lot numbers of the standards used are maintained throughout the course of any study.

A secondary reference standard is a stock standard prepared using the primary reference standard and sorted away for the duration of a study. New secondary standards are prepared for each new study.

6.1.1.a. Isoniazid Secondary Standard Preparation

a. On a calibrated analytical balance, weigh out approximately 10 mg of isoniazid accurately into a clean Grade A 10 ml volumetric flask.
b. Add approximately 9.5 ml distilled deionised water, topping up the flask to the demarcation with a glass Pasteur pipette to produce a final concentration of 1 mg/ml. Vortex well until there is no precipitate remaining.
c. Prepare a set of polypropylene microcentrifuge tubes, suitable to withstand -80°C and 3000 rpm.
d. Aliquot 100 µl of the secondary stock standard into each tube, sealing immediately.
e. Store the set in a labelled cryobox at -80°C, until required for use.

6.1.1.b. Pyrazinamide Secondary Standard Preparation

a. On a calibrated analytical balance, weigh out approximately 40 mg of pyrazinamide accurately into a clean Grade A 10 ml chromated volumetric flask.
b. Add approximately 9.5 ml HPLC grade methanol, topping up the flask to the demarcation with a glass Pasteur pipette to produce a final concentration of 4 mg/ml. Vortex well until there is no precipitate remaining.

c. Prepare a set of polypropylene microcentrifuge tubes, suitable to withstand -80°C and 3 000 rpm.

d. Aliquot 100 µl of the secondary stock standard into each tube using an accurate automatic research pipette, sealing immediately.

e. Store the set at -80°C, until required for use.

6.1.1.c. Rifampicin Secondary Standard Preparation

a. On a calibrated analytical balance, weigh out approximately 10 mg of rifampicin accurately into a clean Grade A 10 ml volumetric flask.

b. Add approximately 9.5 ml HPLC grade methanol, topping up the flask to the demarcation with a glass Pasteur pipette to produce a final concentration of 1 mg/ml. Vortex well until there is no precipitate remaining.

c. Prepare a set of polypropylene microcentrifuge tubes, suitable to withstand -80°C and 3 000 rpm.

d. Aliquot 100 µl of the secondary stock standard into each tube, sealing immediately.

e. Store the set at -80°C until required for use.

6.2. Preparation of Reagents

6.2.1 0.5 mM Potassium phosphate buffer pH 4.5 (Expiry 1 month)

a. Weigh 0.68 g of KH_2PO_4 and dissolve in 1000 ml distilled, deionised water.

b. While solution is stirring adjust the pH to 4.5 with phosphoric acid using a calibrated pH meter

c. The pH of this solution is checked at the beginning of each day and adjusted if necessary.

6.2.2.a. Isoniazid and Pyrazinamide Mobile Phase: 5% Acetonitrile : 95% (0.06%) Trifluoroacetic acid

i. Line A: Acetonitrile

ii. Line B: 0.06% Trifluoroacetic acid (Expiry 1 month)

0.06% Trifluoroacetic Acid:

a. Fill an A grade 2000 ml chromated volumetric flask with distilled, deionised water to the 2 000 ml demarcation.

b. Using a calibrated research pipette, remove 1.2 ml water and discard.

c. Replace the 1.2 ml with 1.2 ml of trifluoroacetic acid

d. Shake well until the schlieren lines disappear.

6.2.2.b. Rifampicin Mobile Phase: 60% Acetonitrile : 40% (0.1%) Trifluoroacetic acid

i. Line A: Acetonitrile

ii. Line B: 0.1 % Trifluoroacetic acid (Expiry 1 week)

0.1% Trifluoroacetic Acid

a. Fill an A grade 2000 ml chromated volumetric flask with distilled, deionised water to the 2 000 ml demarcation.
b. Using a calibrated research pipette, remove 2.0 ml water and discard.
c. Replace the 2.0 ml with 2.0 ml of trifluoroacetic acid.
d. Shake well until the scleren lines disappear.

6.2.3. Quality Control measures:

6.2.3.1. Standard curve and Quality Control Samples (in µg/ml):

Both the standard curves and quality controls are prepared by spiking large volumes of drug-free plasma with standards of rifampicin (1 mg/ml and 100 µg/ml), isoniazid (1 mg/ml and 1 µg/ml) and pyrazinamide (4 mg/ml and 400 µg/ml) and storing the aliquots at -80°C until required for use. Dilutions of the stock standard are diluted immobile phase for each HPLC system. Each stock plasma solution is prepared by removing the appropriate volume of plasma from the 10 millilitre total volume before spiking with drug standard. A blank extraction is run prior to using a batch of human plasma to test for interfering peaks. This chromatogram is retained for future reference, eliminating the need to run multiple blanks during sample analysis. Peak area is plotted against concentration (µg/ml) and the relationship is linear.

The rifampicin calibration curve range is from 0.3 µg/ml up to 25 µg/ml in plasma, the limit of quantification being 0.3µg/ml. Suggested quality control concentrations are 1.0 (Low), 5.0 (Medium) and 10.0 µg/ml (High).

The isoniazid calibration curve range is from 0.2 µg/ml up to 15 µg/ml in plasma, the limit of quantification being 0.2 µg/ml. Suggested quality control concentrations are 0.5 (Low), 5 (Medium) and 10 ug/ml (High).

The pyrazinamide calibration curve range is from 0.2 ug/ml up to 70 µg/ml in plasma, the limit of quantification being 0.2 µg/ml. Suggested quality control concentrations are 0.5 (Low), 30 (Medium) and 60 µg/ml (High).

6.2.3.2. Method of Quantification:

During the validation period, standard curves are run to determine linearity and reproducibility. Concentrations are calculated using the equations resulting from the validation curves, and the curves run during analysis are run to ensure continued linearity, the quality controls run to ensure day-to-day reproducibility and sample analysis homogeneity.

A 10 µg/ml standard in mobile phase of isoniazid and rifampicin and a 30 µg/ml standard in mobile phase of pyrazinamide are run periodically to ensure accurate identification of each analyte.

For each calibration standard, the peak area is determined. A linear regression describing the calibration curve is then calculated using the following regression equation:

$$y = m(x) + c$$

y = absolute peak area of the analyte

x = concentration of the analyte

m = slope of the calibration line

c = y-intercept of the calibration line

The data reported for each curve must include the line equation, the correlation coefficient (r) and the residual sum of squares (Sx.y). See SOP entitled "QC measures during HPLC and LC MS analysis".

6.3. Analytical Procedures

6.3.1 Method of Extraction for isoniazid, pyrazinamide and rifampicin

a. Wash the Bond Elut extraction cartridges twice with 2 ml methanol, twice with 2 ml water and once with 1 ml phosphate buffer, pH 4.5. Ensure that the buffer is completely pulled through the column.

b. Apply 500 µl of sample onto the column.

c. Draw it slowly onto the column and let it stand for ten minutes.

d. Pull through to waste.

e. Wash with 1 ml phosphate buffer, pH 4.5 and wipe the needles clean.

f. Elute into glass tubes as follows.

Acetonitrile:	200 µl	stand for 2 minutes then pull through
	200 µl	stand for 2 minutes then pull through
	100 µl	stand for 1 minute then pull through
Methanol:	200 µl	stand for 2 minutes then pull through
	200 µl	stand for 2 minutes then pull through
	100 µl	stand for 1 minute then pull through

This step incorporates a dilution factor of 2 (applying 500 µl, eluting into 1 000 µl)

g. Remove 400 µl of the eluent into an injection vial for the determination of isoniazid and pyrazinamide and dry down under streaming nitrogen gas

h. Inject 50 µl of the remaining eluent onto the rifampicin HPLC system for analysis.

i. Reconstitute the dried down aliquot with 500 µl isoniazid/pyrazinamide mobile phase [5% acetonitrile : 95% (0.06%) Trifluoroacetic acid]. Vortex. This step incorporates a dilution factor of 1.25.

j. Cap the vial and inject 20 µl onto the isoniazid and pyrazinamide HPLC system for analysis.

6.3.2 HPLC Resolution Conditions

Analyte	Isoniazid and Pyrazinamide	Rifampicin
Analytical Column	Waters Spherisorb C8, 5 µm, 4.6 mm x 250 mm	SphariGrom Octyl, 5 µm, C8, 4 mm x 150 mm
Mobile Phase	5% Acetonitrile : 95% (0.06%) Trifluoroacetic acid	60% Acetonitrile : 40 % (0.1%) Trifluoroacetic acid
Flow Rate	1.5 ml per minute	2 ml per minute
Injection Volume	20 µl full loop	50 µl full loop injection
Column Temperature	Room temperature	Room temperature
Autosampler Temperature	Room temperature	Room temperature
Detection Wavelength	270 nm	270 nm
Range	0.02 a.u.f.s.	0.02 a.u.f.s
Attenuation	8	4
Total run time per sample	11.0 minutes	6 minutes

7. *Results:*

7.1. Assay details.

Typical retention times:	Rifampicin	Isoniazid	Pyrazinamide
	5.3 minutes	2.7 minutes	5.3 minutes
Sample run time:	INH/PZA	11.0 minutes	
	RIF	8.0 minutes	

7.2. Standard Curves

Rifampicin stock – 1 mg/ml Isoniazid stock – 1 mg/ml Pyrazinamide stock – 4 mg/ml

Calibrator No.	*[Isoniazid] µg/ml*	*[Pyrazinamide] µg/ml*	*[Rifampicin] µg/ml*
1	15.00	70.00	25.00
2	13.50	63.00	22.50
3	11.25	52.50	18.75
4	7.50	35.00	12.50
5	2.25	10.50	3.75
6	0.75	3.50	1.25
7	0.20	0.40	0.40
8	0.20	0.20	0.30

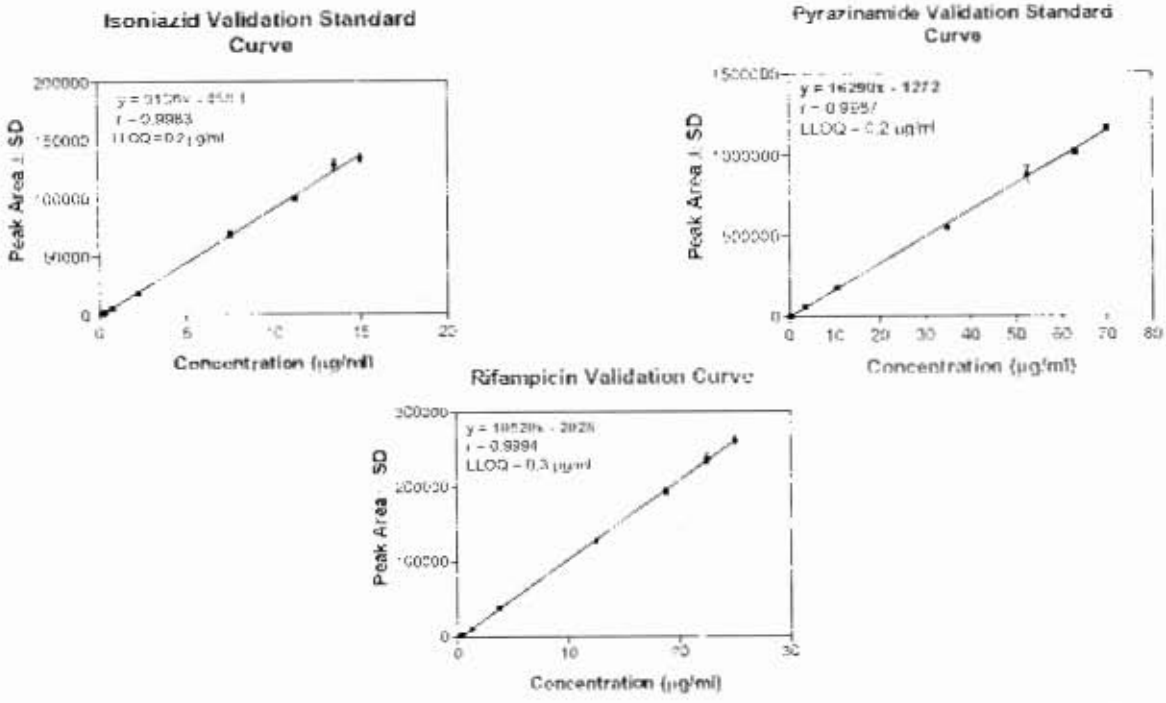


Figure 1. Examples of calibration curves for rifampicin, pyrazinamide and isoniazid.

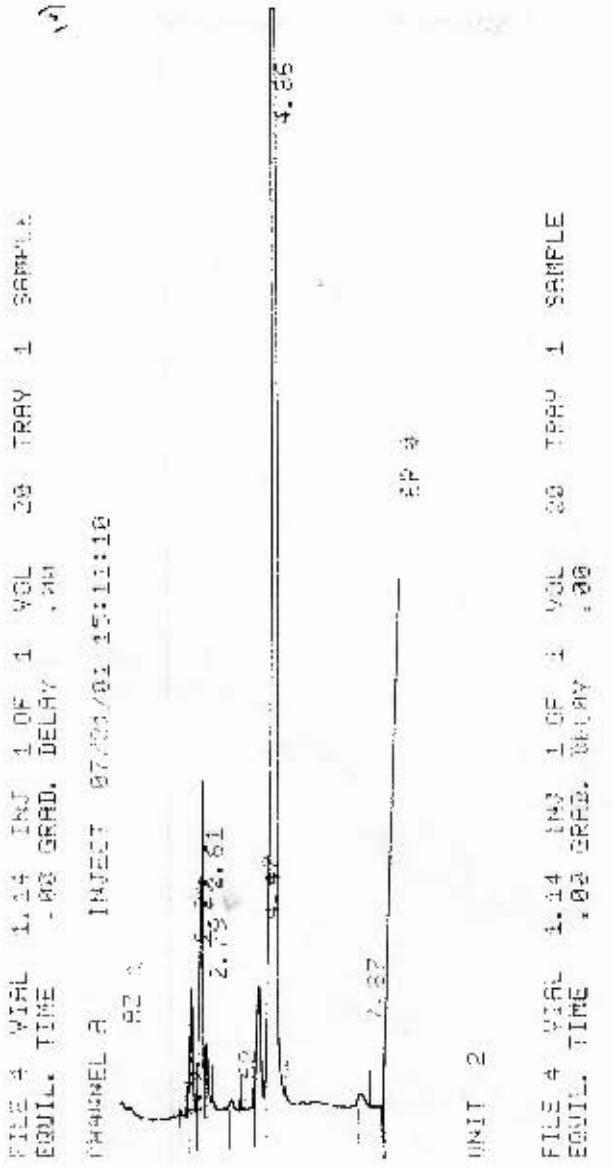


Figure 2b. Low quality control for 2.5 µg/ml isoniazid (2.61 min) and 15 µg/ml pyrazinamide (4.86 min).

appendix 3.2

DETERMINATION OF PLASMA ETHAMBUTOL CONCENTRATIONS

1. **Title: A High Performance Liquid Chromatography Mass Spectrometry Method for the Determination of Ethambutol in plasma**

2. **Scope:** This assay applies to all plasma samples received for ethambutol analysis by the Analytical Laboratory.

3. **Purpose:** To determine the levels of ethambutol in plasma using high performance liquid chromatography coupled to mass spectrometry.

4. **General:** This assay was set up on the Waters/Micromass instrument for analysis. The purpose of this assay is to determine concentrations of ethambutol in plasma samples, primarily for the purposes of clinical studies, comparing fixed dose combinations to single drug preparations.

Specimen Collection: Blood samples are collected in Lithium Heparin coated plastic tubes with gel separator. At least 500µl of blood is required for this assay. The tubes are gently mixed to distribute the anti-clotting factor throughout the blood and put on ice. The tubes are spun down at 3000 rpm for 10 minutes to separate the plasma from the erythrocytes. Plasma is then decanted into labelled tubes and stored at -80°C until required for assay. The stability of ethambutol at -80°C is considerable.

5. **Procedure:**

5.1. ***Reagents:***

The following reagents are required for the assay:

Acetonitrile HPLC grade

Methanol HPLC grade

Deionised reagent grade water (Millipore)

Trifluoroacetic acid (analytical grade)

Ethambutol-free Li[1] anticoagulated human plasma (for calibrators and quality control samples), donated by staff members

Ethambutol hydrochloride – supplied by Sigma.

Neostigmine internal standard - supplied by Sigma

1.2 ml polypropylene microcentrifuge tubes

Ammonium acetate 4mM

5.2. ***Reagent Preparation:***

5.2.1. *Mobile Phase*

Acetonitrile	50%
4 mM Ammonium acetate + 0.1%TFA	50%

5.2.2. *4 mM Ammonium acetate + 0.1% Trifluoroacetic acid (TFA)*

5.2.2.1. Weigh out 0.31 g Ammonium acetate (analytical grade).

5.2.2.2. Make up to 1000 ml with deionised water.

5.2.2.3. Remove 1 ml of solution and add 1 ml TFA.

5.2.2.4. Filter mobile phase using a vacuum filter apparatus.

5.2.2.5. Sonicate each bottle for 10 minutes.

5.2.2.6. Store labelled at room temperature.

5.2.3. *Ethambutol stock standard*	Ethambutol	1 mg
	50% MeOH:50% Water	1 ml

5.2.3.1. Weigh out 1 mg ethambutol.

5.2.3.2. Dissolve in 1 ml 50% MeOH:Water mix.

5.2.3.3. Prepare dilutions of the stock standard to concentrations of 100 µg/ml (1:10); 10 µg/ml (1:100) and 1 µg/ml (1:1000) in mobile phase.

5.2.3.4. Store labelled in 1 ml aliquots at -80°C.

5.2.3.5. Complete the solutions documentation logbook in the weighing room.

5.2.4. *Neostigmine stock standard*	Neostigmine	1 mg
	50% MeOH:50% Water	1 ml

5.2.4.1. Weigh out 1 mg of neostigmine.

5.2.4.2. Dissolve in 1 ml of 50% MeoH:water mix.

5.2.4.3. Prepare a 10 µg/ml dilution of the stock standard by diluting 10 µl stock solution in 990 µl mobile phase.

5.2.4.4. Store labelled in aliquots of 100 µl at -80°C.

5.2.4.5. Complete the solutions documentation logbook in the weighing room.

5.3. Calibrators

Calibrations contain 10 non-zero calibrators. The nominal values are 0.1; 0.2; 0.3; 0.4; 0.5; 1; 2; 3; 4 and 5 µg/ml. A calibration curve is run with every batch throughout the analysis period to check the calibration status of the assay and to calculate concentrations for each sample within the batch. These are made up in ethambutol/neostigmine free plasma samples, obtained from the Western Province Blood Transfusion service at Groote Schuur Hospital.

5.3.1. Calibrator Preparation

Calibrators are routinely made up to 10 millilitres.

Volume of stock µl	Stock concentration µg/ml	Total volume ml	Ethambutol Conc. µg/ml	Cal. No.
100	10	10	0.1	S1
200	10	10	0.2	S2
300	10	10	0.3	S3
400	10	10	0.4	S4
500	10	10	0.5	S5
100	100	10	1.0	S6
200	100	10	2.0	S7
300	100	10	3.0	S8
400	100	10	4.0	S9
500	100	10	5.0	S10

Aliquot the calibrators into labelled 1.2 ml polypropylene tubes – each tube should contain at least 250 µl. Store frozen at approximately -80°C.

5.4. *Quality Control Samples*

Three control samples (nominal concentrations 0.15, 0.45 and 1.5 µg/ml) are used to monitor the performance of assay. It is normal practice to extract all three control samples in duplicate with every set of samples analysed, and to disperse them throughout the run. Control samples should constitute a minimum of 10% of the total batch.

5.4.1. *Quality Control Sample Preparation*

Quality controls are routinely made up to 10 millilitres, using ethambutol/neostigmine free plasma samples, obtained from the Western Province Blood Transfusion service at Groote Schuur Hospital. There are no commercially available quality controls for ethambutol.

Volume of sub stock ml	Stock concentration µg/ml	Total volume ml	Ethambutol Conc. µg/ml	QC No.
150	10	10	0.15	C1
45	100	10	0.45	C2
150	100	10	1.5	C3

Aliquot the controls into labelled 1.2 ml polypropylene tubes – each tube should contain at least 250 ul. Store frozen at -80°C.

5.5. Internal Standard Solution

Prepare a 1 mg/ml stock solution of neostigmine by dissolving 1.0 mg in 1 ml of 50% methanol:water. Dilute the stock solution 1 in 10 (10 µl stock + 990 µl mobile phase) to produce a substock (10 µg/ml).

The stock and substock solutions should be stored at approximately -80°C.

NB: Ensure that there is sufficient working internal standard solution to last a whole calibration curve. Whenever a new working internal standard solution is prepared, the assay will need to be calibrated.

5.6. Extraction Method:

5.6.1. Thaw calibrators and/or controls, as necessary. Thoroughly mix all calibrators, controls and samples. Prepare an assay worksheet in a laboratory notebook in which to record all data.

5.6.2. Pipette calibrator, control or patient sample (200 µl) into a clean polypropylene tube. Using a repeating pipette, add 5 µl of 10 µg/ml neostigmine internal standard solution

5.6.3. Vortex well.

5.6.4. Add 200µl of Acetonitrile.

5.6.5. Vortex well.

5.6.6. Add 200 µl of Acetonitrile.

5.6.7. Vortex well.

5.6.8. Centrifuge at 13 000 rpm for 5 minutes.

5.6.9. Pipette 50 µl of the supernatant into clean dry glass inserts.

5.6.10. Inject 2 µl onto the column for analysis.

5.7. Instrumentation

5.7.1. Start up procedure:

5.7.1.1. Allow the HPLC system to operate and equilibrate the column for 15 minutes before connecting to the mass spectrometer.

5.7.1.2. Inject 5-10 repeats of a 500 ng/ml concentration standard of ethambutol to equilibrate the source block.

5.7.2. Specifications:

Solvent delivery is achieved using a Waters Alliance HPLC system, the pump set at approximately 0.3 ml/minute. The mobile phase consists of acetonitrile:4 mM ammonium acetate + 0.1% TFA (50:50). Sample injection is performed using an auto injector by positive displacement. Chromatography is on a Supelcosil LC-SI 4.6 x 5 mm; 5 µm column maintained at 20°C with a column oven and protected by an inline C18 guard column. Detection is by single quad mass spectrometer.

Mass Lynx software (version 3.5) is used to control the HPLC/MS and record the output from the detector. Integration of peak areas was performed using Mass Lynx software (version no. 3.5) software.

MS Settings: A Waters/Micromass ZMD single quad mass spectrometer equipped with a turbo-ion spray (heated electrospray, ESI) source heated to 350°C is used to introduce the sample into the mass spectrometer. Nitrogen is used as the nebulising gas.

Sample ID	Product Ion (m/z)	Ionisation Mode
Ethambutol	205.2	positive
Neostigmine	223.2	positive

5.8. Assay Acceptance and Reporting of Results

Using the Mass Lynx software (version no. 3.5), integrate the ethambutol and internal standard peak areas for each calibrator, control and unknown sample. Ethambutol concentrations for controls and patient samples will be calculated from the calibration curve. Ethambutol results for unknown samples should be reported to 1 decimal place.

The upper limit of quantification (**ULOQ**) of the LC/MS ethambutol assay is the nominal value of the highest calibrator, namely 5 µg/ml. Any sample whose ethambutol result is calculated to be above 5 µg/ml should be diluted with ethambutol-free plasma and reanalysed. The result for the diluted sample should be multiplied by the dilution factor to give the final result for that sample. This is added to the following ethambutol assay batch.

The lower limit of quantification (**LLOQ**) of the LC/MS ethambutol assay has been set at 0.1 µg/ml. Therefore any sample with ethambutol concentration below the LLOQ should be reported as <0.1 µg/ml.

5.9. Run Acceptance

5.9.1. Calibration Curve Acceptance Criteria:

- Four out of the six calibrators (or two thirds) should be within 15% of their nominal concentration. Twenty percent deviation is allowed for the lower limit of quantification.

5.9.2. Quality Control Acceptance Criteria:

- Two out of the three controls run must be within 15% of their nominal concentration.
- If run in duplicate, two out of the six controls run may fall outside these limits, but both may not be of the same concentration.

NOTE: In the interests of patient care, the laboratory director may release, at his discretion, results that are within the therapeutic range with the added inaccuracy of the controls should they be out of range.

appendix 4.1

IMMUNOLOGY METHOD FOR WHOLE BLOOD CYTOKINE STIMULATION

Method for cytokines: pharmacology project.

- Dilute blood 1 in 2.5 with RPMI 1640 (note final dilution of blood is 1 in 5). Centrifuge remainder of blood. Freeze plasma in 3 aliquots at –20°C for evaluation of IFN-γ, IL-5 and IL-10.
- Plate 100µl per well of diluted blood in a 96-well U-well microtiire plate for 5 days. Plate in triplicate a background, PHA (purified phytohaemagglutinin) and PPD (purified protein derivative of *Mycobacterium tuberculosis*). Plate sufficient wells for IFN-γ, IL-5 and IL-10 (i.e. three x 3 wells for background, 3 wells for PHA and 3 wells for PPD).
- Add mitogen or antigen to the appropriate wells of diluted blood in a 100µl volume. To the PHA wells, add mitogen to ensure a final concentration of 5.75 x 10³MU/ml (MU = mitogenic units). To the PPD wells add antigen to ensure a final concentration of 3µg/ml. To the background wells add 100µl of RPMI 1640.
- Final dilution of blood is therefore1 in 5 with RPMI 1640.
- Incubate for 5 days at 37°C in a humidified atmosphere containing 5% CO_2.
- After 5 days incubation, carefully remove 100µl of supernatant from each well, ensuring that the button is not disturbed. Freeze aliquots at –20°C for evaluation of IFN-γ, IL-5 and IL-10.

Commercially available antibody pairs were used for IFN-γ, IL-5 and IL-10 enzyme-linked immunosorbent assay (ELISA; PharMingen, San Diego, CA) following optimization. Recombinant cytokines were used as standards. Results are expressed in pg/ml after the deduction of unstimulated samples (i.e. background wells).

The lower limit of quantification for the unstimulated samples was 8 pg/ml; background levels below this level assumed a level of 8. The PPD and PHA stimulated levels were calculated by deduction of the levels detected in the background wells from the levels detected in the PPD and PHA stimulated wells. If the result was less than zero; it assumed a value of 0 pg/ml.

appendix 4.2.1

SMEAR MICROSCOPY

Summary of the standard operating procedure used by the SAIMR TB Department at Green Point for tuberculosis microscopic examination

Slides are prepared according to standard operating procedures using direct staining with Ziehl-Neelsen carbol-fuchsin or the flourochrome stain-auramine O method. Positive and negative control slides were used. Special precautions included avoidance of underdecolourization with acid alcohol, avoidance of producing thick smears, precise timing of counterstaining with potassium permanganate to avoid quenching of fluorescing bacilli, reading of fluoro chrome stains while freshly stained to avoid fading, and strict adherence to safety procedures.

Microscopy was preformed according to standard operating procedures, using bright field microscopy or fluorescent microscopy.

For bright field microscopy at least 100 microscopic fields were examined in a systematic manner. When no acid-fast bacilli (AFB) were found in 100 fields, a more thorough search was made in 100 new fields.

The same technique was used for fluorescent microscopy. A maximum of 30 fields were examined at a final magnification of at least 200X.

If an unusual number of smears were positive, potential sources of false positive smears were considered, including contamination of water and water reservoirs with AFB, cross-contamination between slides during the staining process, transfer of AFB in the oil used for oil immersion microscopy, and inexperienced microscopists.

Further limitations noted included:

Organisms other than mycobacteria (e.g. Nocardia spp., Legionella micdadei, and cysts of Cryptosporidium spp. and Isospora spp.) may display various degrees of acid fastness.

Rapidly growing mycobacteria may vary in their ability to retain acid-fast stains; and most will not appear fluorescent on fluoro chrome-stained smears.

appendix 4.2.2

CULTURE OF Mycobacterium tuberculosis

Summary of the standard operating procedure used by the SAIMR TB Department at Green Point for primary isolation of mycobacteria using the Bactec method

Specific quality control procedures included growth of a standardized suspension of H37Rv ATCC27294 in BACTEC 12 B (Middlebrook 7H12) medium, performance testing of the Bactec 460 instrument (Becton Dickenson), and routine instrument maintenance to ensure optimal functioning and avoid cross-contamination between specimens.

Specimens were prepared using standardized decontamination procedures.

Prior to inoculation, the 12B medium containing vials were tested for contamination, by checking the growth index and PANTA (a mixture of the antimicrobials polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) was added to the vial to suppress contamination.

0.5 ml of decontaminated specimen was added to the 12 B –containing vial (with PANTA)

Vials were incubated at 37±1°C. Vials corresponding to specimens with a positive smear were read every 2nd day until positive. Vials corresponding to smear negative specimens were read weekly for 5 weeks.

A growth index (GI) > 20 was considered positive. Vials identified by a growth index > 20 were separated and read daily. A daily increase in the GI of 2-3 X was typical for *Mycobacterium complex* and greater than 3X for other mycobacteria. Once a GI >100 was achieved a smear was prepared from the medium in the vial using smear-fixing solution, and examined under oil immersion. The morphology of the organism was inspected to presumptively discriminate between *Mycobacterium complex* other mycobacteria.

All positive vials were examined for the presence of contamination; if contaminated the culture was reprocessed using a standardized salvage procedure.

Limitations noted:

Colony morphology cannot be observed
Mixed growth of more than 1 kind of mycobacterium cannot be detected
A subculture in a solid medium is needed to isolate a mixed culture.

appendix 4.3

RIFAMPICIN AND ISONIAZID SUSCEPTIBILITY; MINI-MIC METHODS

DRUG SUSCEPTIBILITY TESTING

ALL PROCEDURES TO BE CARRIED OUT IN A BIOLOGICAL SAFETY CABINET

Isolates that are to be processed further for sensitivity testing must fulfil the following criteria.

- Patient must already be on anti-TB therapy and still have a positive culture
- Patient is a recently treated TB (up to one year ago) and has a positive culture again
- On request

Sensitivities and PCR are also done on all isolates submitted from Red Cross Hospital.

Each specimen is numbered, indexed and sent for registering after which the form is filed in the "awaiting sens" file.

The Bactec susceptibility is recommended only for M.tuberculosis

INTRODUCTION

The BACTEC procedure for drug susceptibility testing of mycobacteria is based on the same basic principle employed in the conventional method. The only difference is that a liquid medium is used and instead of counting colonies after about 3 weeks, the growth is monitored radiometrically and the results are reportable within 4 to 5 days. The critical proportion for resistance is taken as 1% for all antituberculosis drugs. This means that if 1% or more of the test mycobacterial population is resistant, the culture is considered resistant for laboratory reporting purposes. Resistance is determined by comparing the rate of growth in the control and the 12B vials containing test drug.

To determine the 1% proportion of resistance, the bacterial inoculum used in the control vial is 100-fold less than that used for the drug-containing vial.

PREPARATION OF DRUG DILUTIONS (when using commercial kit)

ISONIAZID:(0,1µg/ml 12B): reconstitute the lyophilized drug with 5ml sterile distilled water (4µg/ml) and shake well until the drug is completely dissolved. Aliquot 0,15ml amounts of this stock into TEKLAB tubes and freeze at -70°C.

RIFAMPICIN: (2 µg/ml 12B): reconstitute the lyophilized drug with 5ml sterile distilled water (80µg/ml) and shake well until the drug is completely dissolved. Aliquot 0,15ml amounts of this stock into TEKLAB tubes and freeze at -70°C.

WHEN PREPARING INH FROM POWDER

Weigh out 0,001g (1mg) of INH powder (0,001g≡1mg≡1000ug)
Dissolve in 1,56ml water
This solution is the equivalent of 640µg/ml
Make a 1:10 dilution, i.e. 1ml of 640µg/ml + 9ml sterile distilled water = 64µg/ml
Filter sterilize using 0,2µm Millipore filter available from the media lab
Aliquot 1,3ml amounts into Teklab tubes,date and store in the –70°C freezer.
This stock solution has an expiry date of 6 months.

For working solution make a 1:16 dilution of 64µg/ml to obtain 4µg/ml.
I.e.: 1ml INH + 15ml water.
Aliquot 160µl per Teklab tube and freeze. Use 1 tube per sensitivity.

WHEN PREPARING RIF FROM POWDER

Weigh out 0,01g (10mg) of RIF powder (0,001g≡1mg≡1000ug)
Dissolve in 15,6ml methanol
Filter sterilize using 0,2µm Millipore filter available from the media lab
This solution is the equivalent of 640µg/ml
Aliquot 1,3ml amounts into Teklab tubes and store in the –70°C freezer
This stock solution has an expiry date of 6 months.

For working solution make a 1:8 dilution of 640µg/ml to obtain 80µg/ml.
i.e.: 1ml RIF + 7ml water.
Aliquot 160µl per Teklab tube and freeze. Use 1 tube per sensitivity.

PREPARATION OF 12B DRUG MEDIUM

Thaw a tube of frozen stock drug solution. Using a tuberculin syringe add 0,1ml of this stock solution into a 12B vial. Use a separate syringe for each drug. All the 12B vials used in the test should be pre-tested on the Bactec 460 to establish a CO_2 atmosphere in the vial and to screen out any vials with a GI 20 or more.

SPECIAL DILUTING FLUID (0.2% fatty acid free bovine serum albumin-prepared in the media lab)

The test inoculum is diluted before being added into the control 12B vial. A special diluting fluid, which does not compete with the labelled substrate in the 12B medium, is used to assist in dispersing mycobacteria.

PREPARATION OF INOCULUM FROM 12B MEDIUM

Before performing a susceptibility test, confirm the ID of the organism. This will avoid unnecessary susceptibility testing of MOTTS and *M.bovis* BCG's.

- If the GI of the 12B vial is 999 sub 0,05ml of this to an appropriately labelled 12B vial and place this vial in the "awaiting growth for sens" rack.
- Control of the GI vials. The GI must be between 300 –500 before performing a sensitivity test as per protocol

PREPARATION OF INOCULUM FROM MGIT TUBE, including H37RV ATCC 27294

Before performing a susceptibility test confirm the ID of the organism first. This will avoid unnecessary susceptibility testing of MOTTS and *M.bovis* BCG's.
Vortex the MGIT tube

- Inoculate 0,25ml from the MGIT into an appropriately labelled 12B vial.
- Place in the "awaiting growth for sens" rack.
- Control the GI of the vial daily. The GI must be between 300-500.
- When ready conform the sensitivity as per protocol

INOCULATION PROCEDURE

- Prepare drug media by adding 0,1ml of each test drug stock solution into individual 12B vials
- Arrange the vials in a rack and label properly (CONTROL, INH, RIF).
- Prepare a uniform bacterial inoculum, by vortexing the 12B vial. This inoculum is used directly.
- Clean the 12B vial septa with 70% alcohol.
- Using a tuberculin syringe inoculate 0,1ml of the bacterial suspension into each of the 12B vials containing a drug.
- For the control vial do not inoculate directly but first dilute 1:100 by transferring 0,1ml of the suspension into 9,9ml special diluting fluid. After mixing thoroughly inoculate 0,1ml of this dilution, using a new tuberculin syringe, into the control 12B vial (without a drug)
- Incubate all the vials at 37°C. Do not incubate below 36°C.

DAILY READING SCHEDULE (including weekends and public holidays)

- Test vials at the same time each day, usually at 2pm.
- Test for a minimum of 4 days and a maximum of 12. If there is no growth by day 4 in the control vial continue to incubate the vials and test daily until the control vial reaches a GI of 30 or more - to a maximum of 12 days.
- Record each day's readings in the workbook.

INTERPRETATION OF RESULTS

GI readings of a susceptibility test require more interpretation than do the primary isolation vials. For the first two to three days, the GI in the control vial will be low but then will start increasing by a factor of two to three. Since the vials containing drugs were inoculated with a 100-fold larger inoculum, the GI readings are usually higher than the control for the first day or two. If the strain is susceptible to the test drug, the GI levels off or decreases on the subsequent days. However, the GI value continues to increase for resistant strains and is much higher than the control.
This is the reason that a resistant strain can be detected and reported earlier than a susceptible strain

- The difference between the current GI value and the GI value from the previous day is designated ΔGi.
- When the control vial reaches a GI of 30 or more the results should be interpreted as follows:

ΔGI Control > ΔGi drug	susceptible
ΔGI Control < ΔGi drug	resistant

Expected results of control H37RV
The H37RV control should always be sensitive to both INH and Rif

Corrective Action if control is resistant
Check the expiry dates of the drugs. If the drugs have been used beyond the expiry date, make up a fresh batch of drugs.
Retest the H37RV with the newly made drug/drugs, and all samples that were resistant in the batch.

- Record results in the workbook and in the sensitivity file
- Remove the original form from the "awaiting sens" file, record the result on the front of the form with the date, and enter result on computer.
- Place form on the queue
- If specimen is a RXH or TBH one, do all of the above and then place the form on the "other" queue
- When reports are printed send to TBH or RXH
- Any result showing resistance to either one or both drugs tested must be faxed to Karen Sheen at Brooklyn Chest Hospital – fax: 5103898
- Place the "control" 12B vial of INH and Rif resistant patients in the "awaiting growth for 2nd and 3rd line" rack, incubate and check the GI every 2/3 days, until it reaches close to or at 999.
- Fill in a sensitivity request form; make a copy of this form. Staple the copy to the original request form, place in concertina file.
- Send 12B file and form to NPHLS Green Point.
- Fill in the record in the sensitivity index.
- When the results and reference number are returned , enter them onto the computer, and into the TB sens index.
- Staple the result sheet from Green Point to the orginal request from.
- Place on queue for authorization.

TO MAKE A STOCK SOLUTION OF RIFAMPICIN AT 640µg/ml

Weigh out 0.01g (10mg) of RIF powder (0,001g = 1mg = 1000µg)
Dissolve in 0.6ml methanol. Then add 15ml distilled water.
Filter sterilize using a 0,2µm Millipore filter available from the media lab
This solution is the equivalent of 640µg/ml
Double diluting with 3ml amounts is convenient. Dispense the rest into appropriately labelled Teklab tubes and freeze.

Each 12B vial contains 4ml of medium and by adding 0,1ml the dilution factor is 40

Double dilute 640µg/ml to 320µg/ml
Take 0,1ml and add to 12B = 8µg/ml

Or make a 1:8 dilution in sterile distilled water of 640µg/ml to obtain 80µg/ml and continue diluting as indicated below ie: 1ml RIF + 7ml water

Double dilute 320µg/ml to 160µg/ml
Take 0,1ml and add to 12B = 4µg/ml

Double dilute 160µg/ml to 80µg/ml
Take 0,1ml and add to 12B = 2µg/ml

Double dilute 80µg/ml to 40µg/ml
Take 0,1ml and add to 12B = 1µg/ml

Double dilute 40µg/ml to 20µg/ml
Take 0,1ml and add to 12B = 0,5µg/ml

Double dilute 20µg/ml to 10µg/ml
Take 0,1ml and add to 12B = 0,25µg/ml

Double dilute 10µg/ml to 5µg/ml
Take 0,1ml and add to 12B = 0,125µg/ml

Double dilute 5µg/ml to 2,5µg/ml
Take 0,1ml and add to 12B = 0,06µg/ml

Double dilute 2,5µg/ml to 1,25µg/ml
Take 0,1ml and add to 12B = 0,03µg/ml

Double dilute 1,25µg/ml to 0,625µg/ml
Take 0,1ml and add to 12B = 0,015µg/ml

For each dilution:
Dispense 0,15ml into appropriately labelled Teklab tubes and freeze
For use: Take 0,1ml and add this to a 12B vial

TO MAKE A STOCK SOLUTION OF ISONIAZID AT 640µg/ml

Weigh out 0,001g (1mg) of INH powder (0,001g = 1mg = 1000µg)
Dissolve in 1,56ml distilled water
Filter sterilize using a 0,2µm Millipore filter available from the media lab
This solution is the equivalent of 640µg/ml
Make a 1:10 dilution
ie:1ml of 640µg/ml + 9ml sterile distilled water = 64µg/ml.
Double diluting with 3ml amounts is convenient
Dispense the rest into appropriately labelled Teklab tubes and freeze.

Each 12B vial contains 4ml of medium and by adding 0,1ml the dilution factor is 40

Double dilute 64µg/ml to 32µg/ml
Take 0,1ml and add to 12B = 0,8µg/ml

Or make a 1:16 dilution in sterile distilled water of 64µg/ml to obtain 4µg/ml and continue diluting as indicated below:1ml INH + 15ml water → for routine Sensitivities

Double dilute 32µg/ml to 16µg/ml
Take 0,1ml and add to 12B = 0,4µg/ml

FOR MIC's
make a 1:8 dilution in (1+7) Sterile dist H_2O of 64µg/ml INH to obtain a 8µg/ml conc. Double dilute this down to 0,125µg/ml.

Double dilute 16µg/ml to 8µg/ml
Take 0,1ml and add to 12B = 0,2µg/ml

Double dilute 8µg/ml to 4µg/ml
Take 0,1ml and add to 12B = 0,1µg/ml

Double dilute 4µg/ml to 2µg/ml
Take 0,1ml and add to 12B = 0,05µg/ml

Double dilute 2µg/ml to 1µg/ml
Take 0,1ml and add to 12B = 0,025µg/ml

Double dilute 1µg/ml to 0,5µg/ml
Take 0,1ml and add to 12B = 0,0125µg/ml

Double dilute 0,5µg/ml to 0,25µg/ml
Take 0,1ml and add to 12B = 0,006µg/ml

Double dilute 0,25µg/ml to 0,125µg/ml
Take 0,1ml and add to 12B = 0,003µg/ml

For each dilution:
Dispense 0,15ml into appropriately labelled Teklab tubes and freeze
For use: Take 0,1ml and add this to a 12B vial

appendix 5.1

PLASMA CONCENTRATIONS OF RIFAMPICIN, ISONIAZID, PYRAZINAMIDE AND ETHAMBUTOL AT EACH SAMPLE TIME IN EACH SUBJECT

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
1	0.00	0.00	0.00	1.44	0.20
1	0.57		7.04	54.19	1.09
1	1.00	2.06	9.23	72.42	2.16
1	1.52	11.46	6.48	75.25	1.84
1	2.02	0.55	4.50	71.93	1.51
1	2.52	2.49	6.00	60.09	1.32
1	3.00	9.82	4.72	54.30	3.27
1	4.00	7.82	4.96	49.99	2.47
	5.02	3.93	2.96	41.96	1.28
	8.02	1.21	2.07	30.51	0.97
2	0.00	0.00	0.00	2.47	0.33
2	0.55		0.27	2.00	1.09
2	0.98	0.00	0.72	16.90	0.95
2	1.50	0.00	1.35	25.52	0.72
2	2.00	0.00	1.66	31.26	0.91
2	2.52	1.68	3.67	43.31	2.16
2	3.00	2.13	5.12	47.53	3.94
2	4.00	3.74	4.52	45.65	4.26
2	6.05	3.87	2.71	26.94	2.34
2	8.00	1.65	1.93	18.59	1.47
3	0.00	0.00	0.00	1.59	
3	0.50	0.00	1.22	12.70	
3	1.00	1.09	2.28	25.79	
3	1.50	2.11	3.31	35.05	
3	1.98	2.38	3.17	48.80	
3	2.48	3.68	5.47	45.18	
3	3.00	4.26	5.07	47.35	
3	4.00	4.03	4.56	39.21	
3	6.07	2.85	3.30	29.64	
3	8.08	1.28	1.79	20.15	
4	0.00	0.00	0.00	2.03	0.13
4	0.50	0.00	2.19	20.57	0.43
4	1.00	2.89	5.08	37.94	1.12
4	1.50	4.00	5.45	46.35	0.71
4	1.98	4.21	6.33	41.08	0.91
4	2.52	4.52	5.36	38.47	1.30
4	3.00	3.59	4.61	37.36	1.27
4	4.00	2.46	4.39	35.13	1.30
4	6.05	0.74	2.53	28.29	0.63
4	8.02		1.51	18.09	0.38
5	0.00	0.00	0.00	5.52	0.00
5	0.50	0.68	5.94	53.44	1.48
5	1.00	3.80	5.94	55.19	2.80
5	1.50	1.97	4.42	49.58	2.54
5	2.00	0.74	4.21	44.20	1.49
5	2.50	5.00	3.71	45.90	2.28
5	3.00	5.29	3.23	44.73	0.37
5	4.00	3.60	2.94	43.70	4.25
5	6.00	1.60	1.84	30.99	1.98
5	8.00		1.05	25.83	1.11
6	0.00	0.00	0.00	4.31	0.00
6	0.50	0.57	2.04	40.80	0.48
6	1.00	2.20	3.17	41.36	0.91
6	1.50	3.76	4.71	45.51	0.93
6	2.00	3.71	3.72	41.74	1.83
6	2.50	3.18	2.65	39.51	2.87
6	3.18	3.28	2.97	35.37	4.17
6	4.00	1.61	2.31	34.35	3.39
6	6.00	0.85	1.27	29.10	1.48
6	8.00		0.69	23.26	0.96
7	0.00	0.00	0.00	2.58	0.34
7	0.50	0.00	4.42	42.92	0.54
7	1.00	0.00	5.94	60.03	1.55
7	1.50		7.96	40.75	1.82
7	2.00	5.65	8.29	36.76	1.98

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
7	2.50	5.66	7.82	35.52	2.16
7	3.13	6.13	5.98	34.73	1.50
7	4.00	4.51	5.95	42.01	
7	6.03	2.22	4.69	23.39	0.78
7	8.00	1.30	2.92	17.85	0.74
9	0.00	0.00	0.00	4.96	0.00
9	0.50	2.63	8.77	51.26	0.37
9	1.00	8.53	8.64	52.37	1.33
9	1.50	13.59	9.67	52.00	1.97
9	2.00	14.84	7.56	46.89	3.61
9	2.50	13.03	7.05	45.14	5.33
9	3.00	13.16	6.56	42.52	4.93
9	3.98	9.89	3.96	37.61	4.34
9	5.02	5.62	2.53	27.85	1.94
9	8.00	3.67	1.62	24.29	0.37
10	0.00	0.00	1.35	11.12	0.20
10	0.50	0.45	2.53	18.78	1.98
10	1.00	0.80	5.78	42.83	2.94
10	1.52	0.82	5.35	57.70	1.38
10	2.00	1.25	5.49	49.33	2.55
10	2.50	8.56	6.71	53.51	3.59
10	3.03	7.70	5.29	44.76	6.38
10	4.00	6.31	3.96	45.45	4.06
10	6.02	3.00	3.60	32.13	1.71
10	8.02	1.29	2.34	21.16	1.12
11	0.00	0.00	0.00	3.44	0.25
11	0.57	2.21	4.22	30.59	1.82
11	1.03	5.25	5.01	30.56	4.59
11	1.52	6.13	7.17	42.52	4.69
11	2.00	6.74	5.87	44.07	5.31
11	2.55	6.13	5.35	43.84	3.67
11	3.03	4.01	4.93	39.04	3.27
11	4.00	3.04	3.08	35.10	2.32
11	6.05	0.49	1.35	24.25	1.01
11	8.07		1.28	22.98	0.89
13	0.00	0.00	0.00	3.80	0.37
13	0.52	0.00	0.88	16.84	0.67
13	1.02	0.00	3.29	31.24	1.11
13	1.50	0.00	3.13	35.08	0.94
13	2.00		2.62	39.24	1.01
13	2.50	3.98	5.67	43.70	3.35
13	3.00	5.53	4.70	38.74	2.28
13	4.13	4.91	3.96	33.73	5.85
13	6.13	2.54	2.16	27.82	1.44
13	8.07	0.78	2.23	21.04	1.04
14	0.00	0.00	0.00	3.85	0.00
14	0.50	1.01	4.45	44.84	0.23
14	1.00	3.09	5.10	47.66	1.63
14	1.52	3.89	5.56	46.88	2.09
14	2.00	4.15	4.17	41.76	3.22
14	2.50	4.41	4.42	42.40	3.50
14	3.00	4.26	3.97	38.11	3.24
14	4.00	3.02	2.41	33.54	1.76
14	5.98	2.07	1.75	27.00	0.88
14	8.00	1.26	1.04	24.25	0.86
15	0.00	0.00	0.00	2.21	0.73
15	0.50	0.00	7.69	52.95	1.22
15	1.00	0.00	6.09	75.15	2.05
15	1.50	7.03	10.37	79.13	2.67
15	2.00	8.13	10.32	74.64	3.07
15	2.50	7.92	8.23	73.07	3.79
15	3.00	7.43	7.19	67.84	4.85
15	4.00	5.75	5.17	55.98	3.79
15	6.03	3.89	3.35	50.00	2.25
15	8.08	2.10		42.48	1.68

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
15	0.00	0.00	0.00	1.19	0.22
15	0.50		7.45	30.26	1.28
15	1.00	0.47	9.34	42.98	3.16
15	1.50	1.72	8.69	40.69	2.39
15	2.00	1.75	9.28	38.33	4.1
15	2.50	2.54	8.88	34.66	3.64
15	3.00	2.06	7.05	33.43	2.81
15	4.00	0.90	3.33	24.37	1.80
15	5.08		.80	17.34	1.3
15	8.00		2.25	12.31	0.68
17	0.00		0.00	10.67	0.30
17	0.50		13.28	60.76	1.35
17	1.00	3.60	12.37	65.99	2.16
17	1.50	4.03	11.03	65.77	1.77
17	2.00	5.89	10.86	57.48	2.43
17	2.50	5.7	9.43	54.12	3.86
17	3.00	6.41	9.43	49.61	5.78
17	4.00	7.52	9.04	48.55	2.56
17	6.08	4.30	2.41	47.47	1.29
17	8.05	2.35	1.60	34.65	0.94
18	0.00	0.00	0.00	1.87	0.26
18	0.50		5.59	13.10	0.48
18	1.00	2.05	7.47	27.25	1.22
18	1.50	7.42	7.28	37.71	4.49
18	2.02	8.21	7.27	41.14	7.22
18	2.52	7.93	5.67	36.42	2.54
18	3.02	5.54	3.15	36.57	2.31
18	4.03	5.27	5.24	35.93	1.43
18	6.12	2.18	2.81	25.24	0.80
18	8.03	1.05	1.07	18.62	0.67
19	0.00	0.00	0.54	2.51	
19	0.50	0.00	4.90	17.36	
19	1.00	0.76	11.49	46.74	
19	1.50	4.54	10.01	52.10	
19	2.00	6.72	8.31	48.93	
19	2.67	9.04	10.22	48.85	
19	3.02	7.71	7.95	44.20	
19	4.03	4.61	3.66	36.06	
19	6.03	1.73	3.96	28.51	
19	8.00	0.41	3.70	22.07	
20	0.00		1.78	11.24	
20	0.50	8.89	13.89	66.91	
20	1.00	12.34	11.18	65.70	
20	1.50	14.91	9.72	64.25	
20	2.00	3.76	9.47	91.9	
20	2.50	12.02	8.40	57.86	
20	3.02	9.44	6.60	58.48	
20	4.00	7.56	5.59	53.69	
20	6.10	3.75	3.17	44.68	
20	8.05	2.14	2.78	29.70	
22	0.00	0.00	0.00	3.22	
22	0.50	1.21	9.89	49.27	
22	1.00	8.91	5.94	52.66	
22	1.50	10.69	10.32	51.07	
22	2.02	14.04	11.50	54.39	
22	2.52	11.52	4.29	57.63	
22	3.00	9.52	3.09	50.14	
22	4.00	5.19	2.75	42.31	
22	6.10	2.55	3.03	31.64	
22	8.00	1.35	0.00	27.41	
23	0.00	0.00	0.56	15.23	1.39
23	0.50		3.10	34.89	0.00
23	1.00	1.88	3.53	41.10	2.61
23	1.50	5.55	4.34	58.29	9.40
23	2.00	9.21	2.79	59.98	3.03
23	2.50	11.19	6.04	61.24	3.27
23	3.00	12.9	5.89	49.15	3.65
23	4.00	7.00	4.63	53.66	2.56
23	6.00	4.96	3.82	46.87	3.41
23	8.02	3.89	1.32	42.22	2.27

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
24	0.00	0.00	1.55	7.44	0.68
24	0.50	0.00	6.18	29.24	2.05
24	1.00	5.75	5.66	57.09	1.92
24	1.48	1.79	12.12	57.20	4.19
24	2.04	2.44	7.70	53.01	2.12
24	2.50	2.51	5.17	46.95	5.51
24	2.95	1.90	6.89	50.27	2.50
24	4.00	1.85	6.35	44.43	6.75
24	5.98	0.84	4.94	34.88	2.39
24	8.00	0.32	3.56	28.43	1.70
25	0.00	0.00	0.61	10.42	0.35
25	0.50	4.44	10.45	62.80	4.17
25	1.02	6.07	11.75	69.80	4.16
25	1.50	8.7	9.26	67.43	0.33
25	2.00	10.70	8.59	63.33	1.35
25	2.52	11.2	7.97	62.74	3.60
25	3.00	10.40	8.54	56.41	4.96
25	4.00	7.41	5.83	56.45	2.41
25	6.08	5.83	3.71	45.37	4.47
25	8.03	3.68	1.94	35.33	2.77
26	0.00	0.00	0.00	10.95	0.38
26	0.50	0.45	1.55	35.83	1.11
26	1.00	3.65	4.34	50.64	5.00
26	1.50	4.40	6.86	67.96	2.45
26	2.00	4.88	6.22	63.36	1.94
26	2.50	4.79	5.53	60.99	2.94
26	3.00	4.70	5.16	56.73	5.50
26	4.00	3.37	2.84	52.69	5.48
26	6.00	2.17	3.25	45.28	2.00
26	8.02	0.99	2.96	39.22	0.47
27	0.00	0.00	0.20	1.19	0.32
27	0.50	0.00	2.10	8.29	0.97
27	1.00		7.22	40.57	1.62
27	1.50	0.34	6.30	44.96	4.70
27	2.00	0.46	7.70	53.34	2.99
27	2.52	0.52	6.10	44.10	4.30
27	2.95	0.91	7.23	45.95	5.61
27	4.15	0.66	5.10	36.67	4.76
27	6.03	0.56	2.83	27.95	2.21
27	8.02	0.40	1.94	19.22	2.68
29	0.00	0.00		6.85	0.11
29	0.50	1.42	2.13	18.61	0.80
29	1.00	1.92	3.88	41.84	1.00
29	1.52	1.77	6.08	63.67	1.36
29	2.00	1.63	9.47	70.93	2.0
29	2.52	1.45	7.35	68.57	2.10
29	3.00	1.37	7.13	60.59	2.21
29	4.03	1.35	3.08	59.89	3.52
29	6.00	0.40	0.55	44.99	2.02
29	8.07		0.25	34.73	1.29
30	0.00	0.00	0.00	2.63	0.00
30	0.50	0.00	6.74	24.73	1.12
30	1.00	0.43	10.52	57.45	2.81
30	1.50	0.87	8.65	58.51	4.69
30	2.00	0.96	7.58	54.54	3.74
30	2.45	1.40	6.20	48.90	2.56
30	2.95	1.64	4.51	43.96	2.79
30	3.96	1.03	1.99	38.82	1.59
30	6.02	0.66	1.03	27.26	0.85
30	7.97	0.34	0.00	21.17	0.54
31	0.00	0.00	0.83	12.82	0.46
31	0.50	0.00	4.58	59.55	0.96
31	1.00		5.05	65.87	2.02
31	1.50	4.46	6.14	74.57	2.39
31	2.03	10.54	7.41	72.55	3.15
31	2.52	12.11	4.80	65.55	4.15
31	3.00	10.57	3.44	66.68	3.47
31	4.00	5.76	2.90	53.92	3.82
31	6.00	2.47	1.92	36.09	.56
31	8.14	0.71	1.54	45.65	1.11

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
34	0.00	0.00	3.10	2[illegible]	[illegible]
34	0.50		6.17	39.04	1.94
34	1.00	1.26	8.14	50.11	3.40
34	1.50	2.05	7.50	53.46	3.50
34	1.98	2.00	5.90	52.51	3.15
34	2.52	3.58	4.92	41.70	5.04
34	3.03	3.26	5.44	44.35	4.91
34	4.02	2.14	4.15	37.59	3.69
34	5.02	1.06	1.90	28.13	1.58
34	8.00	0.48	1.51	22.79	1.46
37	0.00	0.00	0.27	8.72	0.40
37	0.50	0.00	4.14	21.44	1.35
37	1.00	0.00	5.75	60.84	3.26
37	1.50	0.00	5.24	57.93	4.09
37	1.98		7.04	61.87	9.39
37	2.52		4.26	56.42	5.39
37	3.00	2.94	4.22	57.15	5.03
37	4.02	2.79	2.55	52.14	3.21
37	6.03	1.55	2.05	42.75	1.12
37	8.06	0.45	1.38	33.91	1.05
41	0.00	0.00		3.76	0.53
41	0.68	0.39	4.32	56.21	5.11
41	1.05	2.05	6.34	57.64	7.92
41	1.50	2.00	5.38	50.48	6.87
41	2.00	1.51	4.19	47.13	6.03
41	2.50	1.19	3.89	44.26	3.52
41	3.00	0.88	3.44	39.01	3.27
41	4.00	0.63	2.59	34.84	2.01
41	6.00	0.35	2.62	26.60	1.36
41	7.99		1.79	22.11	1.11
42	0.00	0.00	0.77	6.53	0.90
42	0.71	0.00	2.02	17.15	0.99
42	1.05	0.00	1.29	22.56	1.11
42	1.52		2.70	33.5	1.45
42	2.00		2.88	39.31	1.97
42	2.50		4.52	35.91	2.21
42	3.00		2.58	40.41	2.47
42	4.00		3.34	37.58	3.50
42	6.10	0.71	3.28	30.52	1.62
42	8.00	0.73	2.23	25.74	1.67
43	0.00	0.00	0.24	9.43	0.55
43	0.75	0.00	3.56	32.85	1.66
43	1.08	0.00	4.47	33.51	1.59
43	1.55	0.00	4.13	34.92	1.59
43	2.05	0.00	4.16	37.32	1.62
43	2.60		3.58	35.09	2.86
43	3.00		2.21	26.82	2.00
43	4.00		1.75	30.4	4.56
43	6.10		2.16	26.11	4.17
43	8.24	0.00	0.89	19.43	1.69
44	0.00	0.00	0.39	2.87	0.67
44	0.41	0.00	1.92	36.55	1.56
44	1.00	3.51	9.93	54.48	1.90
44	1.50	10.17	10.20	54.09	1.97
44	2.00	11.60	8.26	51.51	2.42
44	2.50	7.50	5.67	44.07	2.72
44	3.00	8.96	6.76	45.92	2.48
44	4.00	7.01	5.18	40.28	4.56
44	6.00	4.02	3.61	31.53	2.51
44	7.97	2.15	2.55	23.00	1.56
45	0.00	0.00	0.53	7.20	0.47
45	0.55		6.82	35.77	1.21
45	1.02		6.04	44.47	3.00
45	1.50	0.70	5.62	48.54	3.15
45	2.00	1.22	3.46	42.77	3.80
45	2.50	1.42	2.78	39.88	3.94
45	3.00	1.34	2.52	36.77	3.32
45	4.00	1.04	1.76	30.72	2.64
45	6.00	0.41	0.68	20.11	1.08
45	7.97		0.00	4.13	0.76

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
46	0.00	0.00	0.48	13.97	0.63
46	0.50		1.79	32.76	0.99
46	1.00		2.47	31.55	1.26
46	1.50	0.69	2.98	44.42	1.80
46	2.00	2.21	3.70	47.12	2.49
46	2.50	4.67	3.66	48.32	1.89
46	3.00	6.34	4.38	49.89	2.38
46	4.00	3.84	3.42	47.72	5.70
46	6.07	2.6[illegible]	1.57	4[illegible].36	3.05
46	8.06	.09	1.23	35.47	2.27
47	0.00	0.00		4.28	0.76
47	0.48	0.53	3.20	22.42	[illegible].09
47	1.00	2.31	3.30	30.29	1.44
47	1.52	4.81	3.45	35.36	[illegible].18
47	2.00	5.11	3.54	36.18	2.44
47	2.50	5.84	2.92	36.44	3.93
47	3.00	5.35	2.67	34.44	3.53
47	4.00	3.89	2.23	31.97	4.05
47	6.05	2.06	1.31	24.81	1.81
47	8.08	1.05	0.72	22.63	0.90
48	0.00	0.00		11.79	0.52
48	0.50		.25	29.97	0.66
48	1.00	3.01	1.82	55.27	1.11
48	1.50	0.84	2.41	58.97	1.17
48	2.00	1.23	4.12	59.40	
48	2.50	1.64	3.64	60.09	2.21
48	3.00	2.51	3.32	58.72	2.77
48	4.00	3.24	1.98	52.53	4.79
48	6.08	2.65	1.58	46.11	1.31
48	8.02	2.69	0.92	40.18	0.72
49	0.00	0.00	0.00	5.49	0.46
49	0.50		1.19	18.7	1.38
49	1.00	1.35	2.84	37.00	4.18
49	1.50	1.78	2.96	39.19	5.25
49	2.00	1.62	3.16	41.56	3.11
49	2.52	1.30	1.69	27.68	3.46
49	3.00	1.13	2.71	36.99	2.95
49	4.00	0.61	1.62	34.12	2.74
49	6.06		0.75	27.96	.16
49	8.00		0.35	22.74	0.34
50	0.00	0.00	0.28	8.60	0.45
50	0.50	0.47	15.04	9.84	1.97
50	1.00	17.43	10.55	78.03	2.26
50	1.50	19.32	8.36	77.09	2.57
50	2.00	20.7	7.66	73.58	3.00
50	2.52		5.15	62.82	5.69
50	3.05	12.04	3.95	59.14	3.35
50	4.00	9.37	2.99	56.38	6.24
50	6.00	6.24	2.06	46.47	7.09
50	8.02	4.57	1.04	38.90	1.59
51	0.00	0.00		5.92	0.41
51	0.50	1.20	5.38	59.18	1.58
51	1.00	.65	3.95	59.34	1.20
51	1.50	2.24	3.50	56.77	1.42
51	2.00	2.36	2.70	53.79	2.12
51	2.52	2.68	2.46	52.36	2.70
51	3.00	4.12	2.32	50.77	3.28
51	4.00	4.35	1.46	44.28	4.62
51	6.00	2.49	0.00	37.09	2.30
51	8.00	1.49	0.00	31.17	1.23
52	0.00	0.00	0.00	13.96	2.02
52	0.53		0.61	23.90	2.67
52	1.00	3.6	1.52	38.18	2.14
52	1.50	4.41	2.26	40.28	2.91
52	2.00	5.11	2.09	34.28	3.09
52	2.52	8.21	1.64	38.16	4.48
52	3.00	8.11	1.47	37.35	3.64
52	4.00	7.00	1.04	35.59	3.16
52	6.00	3.53	0.60	32.20	6.61
52	7.97	2.67	0.00	28.39	5.84

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
53	0.00	0.00	5.06	3.24	0.41
53	0.55	1.35	6.65	16.81	1.50
53	1.05	3.02	0.74	26.64	0.82
53	1.55	3.26	0.78	26.43	1.41
53	2.05	2.42	0.70	26.00	1.16
53	2.53	4.26	0.76	19.61	1.71
53	3.00	4.62	6.65	24.79	3.24
53	4.02	7.63	0.63	21.53	2.98
53	6.00	1.12	0.50	16.90	1.43
53	8.05	0.40	0.00	16.64	1.07
54	0.00	0.00	0.00	3.78	0.25
54	0.52	0.00	5.39	23.47	1.20
54	1.00	0.00	4.60	34.67	0.78
54	1.50	0.00	6.28	40.66	1.42
54	2.02	0.00	6.39	50.63	1.16
54	2.52	0.00	5.73	56.50	1.42
54	3.00	0.00	3.74	49.02	1.61
54	4.02	0.50	2.66	33.03	1.76
54	6.00	0.00	1.58	27.46	1.12
54	8.00	0.00	0.63	9.00	0.29
55	0.00	0.00	0.00	3.52	0.24
55	0.52	0.00	1.38	0.00	1.31
55	1.01	2.02	3.32	15.52	3.67
55	1.52		4.63	43.25	4.73
55	2.00	0.45	7.73	50.65	3.72
55	2.52	11.63	7.95	29.80	2.83
55	3.00	6.21	4.50	27.78	2.48
55	4.00	6.26	3.25	29.27	2.19
55	6.00	2.82	1.49	27.53	1.18
55	8.00	1.07	0.75	19.19	0.82
56	0.00	0.00	0.00	2.01	0.28
56	0.50	3.64	9.20	73.50	1.41
56	1.00	6.54	7.75	72.03	4.33
56	1.50	5.55	7.33	80.33	3.87
56	2.00	6.98	7.80	69.65	4.96
56	2.50	7.12	7.09	65.04	5.13
56	2.98	5.51	4.84	51.19	3.58
56	4.02	2.66	4.55	33.01	1.91
56	6.02	2.32	3.06	43.15	1.02
56	8.00	0.91	1.88	33.07	0.36
57	0.00	0.00	0.29	3.40	0.13
57	0.50	0.00	5.12	25.92	0.53
57	1.00	0.00	6.17	39.98	1.85
57	1.50	0.25	6.97	52.67	2.89
57	1.98	3.54	6.59	58.20	3.83
57	2.50	4.57	5.08	49.18	1.74
57	2.98	4.52	5.22	48.06	1.91
57	4.00	3.17	4.34	26.55	1.72
57	6.02	1.42	0.39	22.56	0.74
57	8.00	0.61	0.47	17.00	0.81
58	0.00	0.00	0.00	2.95	0.47
58	0.52	0.00	3.32	11.21	0.52
58	1.02	1.62	5.09	51.19	1.62
58	1.48	4.15	4.49	60.21	1.20
58	2.00	3.02	5.29	57.81	1.87
58	2.50	4.72	4.28	54.12	2.02
58	3.00	2.84	3.98	46.58	2.21
58	4.02	2.59	6.93	61.39	2.14
58	6.14	2.06	3.41	65.49	0.96
58	8.00	0.76	2.27	36.79	0.65
59	0.00	0.00	0.00	2.59	0.41
59	0.53	0.00	6.24	27.12	1.09
59	1.03	2.00	8.51	47.03	1.88
59	1.48		8.43	49.12	1.54
59	2.00		7.04	55.19	3.27
59	2.50	0.45	7.32	60.26	3.70
59	3.00	0.41	6.79	58.23	3.22
59	4.00	0.60	4.80	46.07	3.04
59	6.03		2.25	41.10	1.41
59	7.96		2.08	33.77	0.69

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
60	0.00	0.00	5.06	3.21	0.43
60	0.50	0.00	2.97	15.58	0.67
60	1.00	0.00	3.69	28.52	0.78
60	1.50	0.00	4.70	37.28	1.11
60	2.00		5.22	47.06	1.15
60	2.50		5.87	52.72	1.71
60	3.00		5.18	48.23	1.97
60	4.00		5.10	46.79	4.25
60	6.00		3.37	38.58	2.17
60	8.00		2.35	30.68	1.38
61	0.00	0.00	0.00	4.06	0.80
61	0.50		2.12	19.45	0.42
61	1.00	0.45	4.55	28.89	1.69
61	1.50	1.03	3.47	30.95	2.06
61	2.00	1.47	4.21	30.33	1.06
61	2.50	2.04	3.37	29.03	2.51
61	3.02	1.89	3.53	27.47	1.88
61	4.00	1.32	1.81	24.13	1.62
61	6.06		1.18	19.73	0.69
61	8.10		0.40	16.19	1.06
63	0.00	0.00	0.00	5.41	0.29
63	0.50	1.41	6.18	47.86	1.15
63	1.00	2.09	4.32	44.30	1.21
63	1.50	2.91	4.06	45.27	1.85
63	1.98	5.96	3.99	51.13	2.33
63	2.48	4.03	3.43	47.98	2.89
63	2.99		2.61	46.21	3.34
63	3.98	4.63	1.73	41.65	4.40
63	6.1	2.06	0.91	33.79	1.65
63	8.1	1.43	0.41	27.79	1.54
64	0.00	0.00		5.62	0.73
64	0.50	3.24	7.55	60.60	2.54
64	1.02	4.56	6.07	53.91	7.22
64	1.50	2.99	4.89	50.69	7.03
64	2.00	3.20	5.07	46.59	4.57
64	2.50	3.10	4.44	48.66	4.87
64	3.00	2.30	3.73	39.14	4.09
64	4.00	1.69	2.30	39.10	2.86
64	6.00	0.89	1.58	31.88	1.83
64	8.00	0.39	1.26	25.48	1.74
65	0.00	0.00	0.00	4.35	
65	0.50		13.26	87.93	2.63
65	1.00	0.81	8.85	64.63	5.53
65	1.50	0.79	7.11	57.30	6.73
65	2.00	0.47	6.10	52.84	4.52
65	2.50	0.40	5.33	48.48	2.94
65	3.00	0.35	3.82	44.38	1.85
65	4.00	0.34	3.56	40.14	1.60
65	6.00	0.52	1.61	32.26	0.65
65	8.00	0.50	0.69	23.52	0.67
66	0.00		0.00	13.36	0.48
66	0.50	1.98	4.00	38.48	1.54
66	1.02	6.50	4.79	51.15	1.99
66	1.52	7.16	4.08	53.19	2.51
66	2.02	10.65	4.12	56.32	3.16
66	2.52	7.08	2.84	56.52	4.13
66	3.00	10.06	2.40	57.69	6.41
66	4.00	7.04	1.52	45.84	5.49
66	6.00	4.53	0.77	37.22	2.96
66	8.05	3.3	0.46	31.70	2.12
67	0.00	0.00	0.00	5.44	1.28
67	0.50	0.50	0.89	20.80	4.25
67	1.00		2.71	36.54	7.45
67	1.55	0.45	3.86	47.24	8.51
67	2.03	1.14	4.40	48.39	3.94
67	2.52	2.83	4.13	47.19	7.71
67	3.00	0.21	3.17	46.16	3.44
67	4.00	1.83	1.83	42.26	9.06
67	6.00	0.41	1.16	32.96	2.68
67	8.05		0.56	24.26	2.03

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	0.00	0.00	0.00	[illegible]	[illegible]
68	0.50	0.00	1.92	14.60	0.86
68	0.98	0.00	3.34	32.19	[illegible]
68	1.50	0.00	3.87	44.16	[illegible]
68	[illegible]		[illegible]	[illegible]	[illegible]
68	2.71		4.66	50.59	3.79
68	3.66		3.75	42.10	4.14
68	4.02	0.57	2.72	44.69	3.45
68	6.03	0.57	[illegible]	34.70	[illegible]
68	8.05	0.77	0.61	7[illegible]8	0.36
70	0.00	0.00	0.00	3.5[illegible]	0.74
70	0.50	0.00	5.21	14.59	1.02
70	1.00	0.00	0.56	50.60	2.04
70	1.50		9.94	60.02	2.25
70	2.00		9.67	52.23	2.18
70	[illegible]		9.40	[illegible]	[illegible]
70	3.06		7.20	40.88	3.83
70	4.03		6.43	37.14	1.45
70	6.00	0.00	3.87	26.72	0.67
70	8.03	0.00	3.12	22.15	0.41
7[illegible]	0.00	0.00	0.00	0.54	0.52
71	0.50	0.00	1.24	4.24	0.58
71	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
71	1.50	0.00	3.76	14.56	2.47
71	2.02	0.00	4.92	15.75	3.04
71	2.52	0.00	5.91	20.4	3.[illegible]
71	3.06	0.00	6.71	10.[illegible]	3.18
71	4.03		3.92	22.93	3.84
71	6.02	0.76	2.69	8.87	1.85
71	8.02	3.33	1.45	10.98	0.55
72	0.00	0.00	0.00		0.67
72	0.50	0.92	10.23	31.65	1.83
72	1.00	3.47	8.16	24.82	2.88
72	1.50	8.43	7.35	22.30	3.13
72	2.00	7.40	7.31	22.82	4.99
72	2.50	7.60	5.98	24.75	4.92
72	3.05	4.77	5.80	18.40	2.74
72	4.00	4.27	4.19	16.80	
72	6.07	1.75	2.47	12.77	
72	8.09	0.76	1.48	9.55	
73	0.00	0.00	0.00	1.64	0.49
73	0.50	0.00	8.00	39.12	1.27
73	1.00		7.95	53.94	1.36
73	1.50	0.35	5.64	50.93	3.91
73	2.02	0.59	6.51	38.78	3.62
73	2.50	1.12	4.30	40.44	4.35
73	3.05	0.71	2.52	27.53	1.09
73	4.05	0.71	3.49	35.53	4.64
73	6.05	0.30	1.57	23.04	2.01
73	8.05		1.18	18.18	0.67
74	0.00	0.00	0.00	2.77	0.71
74	0.50	0.00	5.22	31.09	1.14
74	1.00	0.00	5.69	36.41	1.81
74	1.50	0.89	9.40	48.46	2.30
74	2.00	2.10	7.57	47.41	3.79
74	2.52	3.49	7.95	51.14	3.52
74	3.05	4.57	6.34	42.44	4.46
74	4.06	5.40	5.64	40.24	3.50
74	6.06	1.20	3.17	29.72	2.13
74	8.06		2.06	25.69	1.42
75	0.00	0.00	0.00	5.36	2.09
75	0.48	0.00	4.08	27.51	2.87
75	1.00	1.77	8.06	55.20	5.47
75	1.52	4.76	5.96	49.06	4.89
75	2.00	2.90	5.05	39.03	3.82
75	2.48	2.91	5.86	45.98	3.73
75	3.02	2.61	4.66	41.26	5.42
75	4.05	2.04	4.74	47.61	1.86
75	6.00	1.03	2.36	32.61	1.12
75	8.03	0.35	1.29	23.01	1.14

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	0.00	0.00	0.00	[illegible]	0.45
77	0.50	3.45	9.78	68.10	1.60
77	1.00	[illegible]	8.54	70.98	37
77	1.55	7.22	6.50	60.97	2.10
77	2.00	[illegible]	7.57	[illegible]	[illegible]
77	2.53	6.06	6.02	55.24	5.21
77	3.02	5.90	5.21	[illegible]	4.92
77	4.00	5.34	4.03	45.58	2.08
77	6.05	2.14	2.52	36.54	.60
77	8.00	0.88	[illegible]	28.95	1.00
80	0.00	0.00	0.41	4.52	0.38
80	0.52	0.00	1.69	[illegible]	0.66
80	1.00	0.00	2.01	19.71	1.18
80	1.52		2.59	25.38	[illegible]
80	2.00	0.44	3.97	37.28	1.68
80	[illegible]	[illegible]	4.85	[illegible]	2.50
80	2.98	8.97	3.98	47.91	2.78
80	3.98	5.57	2.85	40.15	2.67
80	6.02	3.29	1.99	31.87	1.55
80	8.03	1.97	1.26	27.54	1.04
81	0.00	0.00		7.09	0.37
81	0.48	0.87	2.38	24.54	1.72
81	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
81	1.50	10.31	5.12	47.46	3.59
81	1.98	11.58	4.69	48.06	4.55
81	2.48	10.70	4.44	47.30	3.81
81	[illegible]	8.60	3.49	[illegible]	[illegible]
81	3.98	7.63	2.77	39.17	[illegible]74
81	6.00	4.31	[illegible]	33.41	1.42
81	8.03	2.36	1.61	24.66	1.27
83	0.00	0.00	0.00	1.73	0.38
83	0.50	0.76	5.34	52.55	1.54
83	1.00	2.62	3.98	56.23	2.60
83	1.48	2.59	3.69	52.20	3.[illegible]
83	2.00	2.51	3.31	47.75	4.09
83	2.50	2.11	2.20	[illegible]	2.30
83	3.00	2.30	1.94	38.29	3.92
83	4.00	1.47	1.77	30.24	3.32
83	6.00	0.55	1.26	24.19	1.73
83	8.00		0.92	17.65	[illegible]7
84	0.00	0.00	0.37	[illegible]	
84	0.50	7.51	3.96	50.29	
84	1.00	12.45	3.92	50.07	
84	1.50	10.93	3.59	51.14	
84	2.00	10.17	4.62	49.87	
84	2.50	10.42	2.64	44.84	
84	3.00	11.20	2.36	45.03	
84	4.00	6.18	2.97	40.11	
84	6.00	3.12	2.22	35.40	
84	8.00	1.77	1.41	29.57	
86	0.00	0.00	0.00	4.30	0.78
86	0.50	0.00	5.67	26.76	[illegible]
86	1.02	0.00	6.85	47.75	2.35
86	1.50		6.11	52.52	1.84
86	2.02	1.02	4.74	56.43	1.15
86	2.50	2.47	5.10	52.30	2.14
86	3.03	3.58	3.84	50.45	2.13
86	4.00	3.51	2.39	42.56	1.46
86	6.05	2.38	1.57	32.47	0.70
86	8.00	0.81	1.41	23.34	0.66
87	0.00	0.00	0.00	0.00	
87	0.53	0.00	0.49	1.47	
87	1.00	0.00	0.67	1.[illegible]	
87	1.50	0.00	0.94	1.42	
87	2.02	0.00	1.37	1.28	
87	2.55	0.00	0.24	1.35	0.[illegible]
87	3.00	0.00	0.30	1.25	0.15
87	4.00	0.00	0.25	1.18	
87	6.13	0.00	0.20	.01	0.[illegible]
87	8.05	0.00		0.63	0.00

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	0.00	0.00	0.00	10.07	0.71
88	0.50	0.71	5.12	66.71	1.44
88	1.00	8.25	6.25	78.61	3.06
88	1.50	13.97	7.46	72.09	4.55
88	2.00	12.92	6.96	63.80	4.35
88	2.52	10.23	3.61	61.39	8.37
88	3.02	7.44	4.32	59.10	8.37
88	4.00	5.11	2.00	53.24	4.00
88	6.00	2.98	1.31	46.66	2.42
88	8.00	1.89	1.58	38.66	1.67
89	0.00	0.00	0.00	5.95	0.41
89	0.50	0.00	3.46	16.03	1.67
89	1.02	0.00	3.73	32.70	1.87
89	1.50	0.00	4.16	43.70	1.90
89	2.00		4.30	47.76	1.13
89	2.50	1.36	4.09	50.42	2.66
89	3.00	3.10	4.48	50.66	3.52
89	4.00	5.55	3.57	47.46	4.47
89	6.06	3.35	2.47	36.91	1.26
89	8.02	1.42	1.98	28.79	0.51
90	0.00	0.00	0.00	11.02	0.49
90	0.50		4.23	41.10	1.24
90	1.00	0.45	3.05	47.80	2.34
90	1.50	0.56	4.50	58.07	3.14
90	2.02	1.76	4.00	64.52	2.67
90	2.50	2.38	4.19	55.10	2.56
90	3.00	3.32	3.21	50.72	2.40
90	4.00	6.26	2.89	51.77	4.50
90	6.03	2.71	2.39	43.67	1.07
90	8.07	0.96	1.75	37.17	1.50
91	0.00	0.00	0.23	5.44	0.50
91	0.50	0.00	2.06	12.64	1.05
91	1.00	0.00	3.75	27.64	2.19
91	1.50	0.00	4.5	32.89	2.39
91	2.00		5.83	36.76	3.56
91	2.50		8.02	71.73	3.47
91	3.00	0.42	7.67	59.47	3.48
91	4.00	3.6	6.31	53.18	2.44
91	6.03	1.54	4.1	45.75	2.76
91	8.00	1.02	3.78	36.90	.78
92	0.00	0.00	2.00	4.49	0.54
92	0.50	0.00	3.14	27.90	0.77
92	1.00		4.11	51.30	1.26
92	1.50	1.69	4.74	47.07	1.64
92	2.00	1.92	4.29	46.84	2.59
92	2.50	2.40	1.63	18.00	2.39
92	3.00	2.23	1.27	19.61	3.05
92	4.00	1.16	0.63	10.11	3.04
92	6.00	0.50	0.73	13.05	1.08
92	8.03		0.00	9.85	0.50
93	0.00		0.77	13.13	1.24
93	0.50		2.03	24.27	1.52
93	1.00	0.85	5.39	50.76	2.54
93	1.50	4.43	6.59	81.97	5.20
93	2.00	5.63	6.64	79.72	4.81
93	2.52	4.98	6.80	78.65	5.31
93	3.00	4.16	4.85	74.91	6.66
93	4.00	5.73	6.76	66.88	4.86
93	6.00	5.75	4.59	57.90	3.46
93	8.00	4.55	3.29	51.16	2.28
94	0.00	0.00	0.00	6.03	0.20
94	0.50	0.00	4.93	28.13	.24
94	1.00	0.00	5.46	53.12	1.23
94	1.50	0.48	5.06	70.19	1.59
94	2.00	2.33	6.05	68.24	2.31
94	2.52	3.63	5.80	66.37	4.33
94	3.02	2.21	5.10	60.52	4.06
94	4.02	1.77	4.14	52.77	3.53
94	6.00	0.88	2.37	44.32	1.19
94	8.00	0.53	0.00	33.64	0.44

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
95	0.00	0.00	0.00	0.42	0.54
95	0.50		10.09	42.40	3.07
95	1.00		8.01	65.03	3.74
95	1.50	0.59	7.23	54.43	3.89
95	2.02	0.79	7.56	57.48	3.75
95	2.53	0.68	6.53	53.58	6.27
95	3.03	0.60	5.05	45.65	6.46
95	4.05	0.42	5.27	39.54	3.37
95	6.00		2.07	28.69	2.06
95	8.00		1.99	23.41	1.50
96	0.00	0.00	0.00	1.95	0.27
96	0.50		0.14	22.32	1.15
96	1.06	2.08	5.44	36.97	2.08
96	1.50	5.45	3.50	39.04	2.73
96	2.00	6.03	2.90	40.50	3.49
96	2.52	7.87	3.82	31.54	3.67
96	3.00	5.51	3.53	27.91	1.37
96	4.00	4.28	2.52	26.08	1.89
96	6.00	1.62	2.36	19.69	1.04
96	8.00	0.38	1.59	14.95	0.77
97	0.00	0.00	0.00	11.15	0.31
97	0.55		3.49	18.10	1.20
97	1.00		3.54	32.45	1.62
97	1.50	1.03	4.32	48.10	1.53
97	2.00	9.84	8.31	50.81	2.42
97	2.50	13.11	6.52	54.61	4.45
97	3.00	8.64	5.37	59.55	5.30
97	4.00	5.60	2.22	42.97	4.65
97	6.00	1.94	1.54	36.90	2.16
97	8.00	0.55	1.69	34.36	1.01
101	0.00	0.00	0.00	4.13	0.55
101	0.50	2.61	1.35	27.13	2.52
101	1.02	3.21	8.96	63.00	3.36
101	1.50	2.09	7.02	62.78	1.99
101	2.00	4.04	7.79	53.07	6.11
101	2.50	2.71	6.45	51.89	4.32
101	3.00	2.83	5.80	47.06	5.71
101	4.00	3.47	4.30	36.03	4.66
101	6.00	0.42	2.74	27.77	2.96
101	8.00		1.76	25.75	1.47
103	0.00	0.00		3.35	0.36
103	0.55	0.55	6.76	35.40	1.05
103	1.05	2.45	6.13	41.61	.88
103	1.53	3.79	5.58	41.51	2.13
103	2.02	5.71	5.75	44.55	4.72
103	2.50	4.49	4.25	40.73	5.96
103	3.00	4.7	4.31	40.40	4.6
103	4.00	3.44	3.22	38.28	2.62
103	6.00	1.70	2.7	29.79	0.62
103	7.97	0.86	1.34	25.63	0.97
104	0.00	0.00	0.00	2.27	0.60
104	0.58	0.00	2.60	13.66	2.91
104	1.00		0.30	23.74	2.21
104	1.52	3.14	5.23	35.21	5.76
104	2.00	4.41	7.36	46.96	5.41
104	2.50	5.31	8.61	69.34	5.64
104	3.00	3.96	6.61	45.95	4.15
104	4.00	3.91	6.52	41.44	2.21
104	6.00	1.60	2.67	29.85	1.49
104	8.00	0.72	1.20	24.59	1.02
105	0.00	0.00	0.00	2.14	
105	0.53	0.00	0.00	23.05	
105	1.00			42.4	
105	1.52			52.94	
105	2.00		8.35	50.46	
105	2.50	3.95	6.61	42.39	
105	3.00	5.35	8.93	40.63	
105	4.02	3.79	6.33	32.56	
105	5.98	1.1	1.86	24.89	
105	8.00	0.32	0.53	20.47	

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
106	0.56	0.00	0.93	31	0.83
106	1.02	0.00	1.86	27.58	0.79
106	1.50	0.00	2.81	28.8	0.69
106	2.02	0.00	4.09	36.76	0.89
106	2.57	0.00	4.42	42.71	1.00
106	3.02		5.26	47.85	1.32
106	4.02	1.04	5.59	44.79	5.09
106	6.00	1.07	3.95	32.50	3.98
106	8.02	0.37	2.49	29.04	2.65
107	0.00	0.00	0.00	3.46	
107	0.50	1.67	2.38	38.19	
107	1.00	3.81	6.36	65.58	
107	1.50	5.67	9.30	60.49	
107	2.00	5.33	8.77	56.79	
107	2.50	5.13	8.51	47.65	
107	3.00	4.31	7.16	48.40	
107	4.02	3.28	5.64	38.90	
107	6.00	1.97	3.19	29.37	
107	8.00	1.13	1.88	22.36	
108	0.00	0.00	0.00	2.44	0.35
108	0.55			54.70	1.68
108	1.10	1.28	2.13	59.25	1.73
108	1.50	3.24	5.40	50.31	2.07
108	2.00	4.17	6.96	46.50	2.72
108	2.50	5.19	8.66	44.81	4.50
108	3.00	4.28	7.13	41.50	
108	4.00	3.27	5.79	34.43	3.31
108	6.00	1.27	2.12	25.96	1.76
108	8.00	0.46	0.77	19.56	1.08
109	0.00	0.00	0.00	5.75	0.40
109	0.53	0.39	0.65	34.60	1.54
109	1.02	2.69	4.49	39.36	2.46
109	1.50	3.65	6.08	51.46	2.70
109	2.00	3.40	5.67	52.23	2.62
109	2.50	3.15	5.26	41.32	3.72
109	3.00	2.37	3.95	46.84	3.79
109	4.00	1.81	3.02	41.48	4.06
109	6.00	0.51	0.85	32.91	2.04
109	8.00		0.22	26.11	1.25
110	0.00	0.00	0.00	1.56	0.50
110	0.54	0.45	0.75	51.74	2.27
110	1.16	1.14	1.91	53.50	3.97
110	1.50	1.48	2.47	54.99	4.12
110	2.05	2.26	3.77	52.00	4.53
110	2.55	2.64	4.41	48.25	3.79
110	3.00	2.06	3.43	46.08	2.63
110	4.00	1.74	2.90	39.67	1.84
110	6.06	1.00	1.66	28.47	1.32
110	8.05	0.52	0.87	20.72	0.92
111	0.00	0.00	0.00	2.35	1.00
111	0.53		0.37	33.56	2.21
111	1.00		0.39	40.24	2.39
111	1.50	1.26	2.14	46.97	2.68
111	2.03	2.10	3.50	45.33	3.61
111	2.50	2.67	4.45	44.65	4.52
111	3.00	4.48	7.47	39.45	2.07
111	4.00	3.25	5.09	35.51	3.64
111	6.00	0.87	1.25	22.99	1.67
111	8.00	0.37	0.62	22.02	1.14
112	0.00	0.00	0.00	5.35	1.11
112	0.58	1.00	1.67	29.31	0.36
112	1.08	2.27	3.78	42.74	1.20
112	1.50	3.86	6.44	43.95	3.48
112	2.00	3.53	5.98	41.93	4.22
112	2.53	4.83	8.06	45.25	4.70
112	3.02	3.88	6.47	40.72	3.81
112	4.00	3.13	5.17	36.79	2.70
112	6.00	1.98	3.25	30.48	1.33
112	8.00	0.83	1.65	25.14	2.72

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
113	0.00	0.00	0.00	5.72	0.40
113	0.52	4.36	7.27	52.00	2.22
113	1.00	5.83	9.72	55.94	2.51
113	1.50	5.83	9.72	59.88	2.57
113	2.00	5.80	9.67	54.51	3.00
113	2.50	5.80	9.67	54.51	1.81
113	3.00	4.20	7.01	47.50	4.79
113	4.00	2.75	4.59	43.20	4.74
113	6.00	1.44	2.40	29.92	3.68
113	8.00	0.56	0.94	31.33	1.84
115	0.00	0.00	0.00	8.10	0.66
115	0.53	0.81	1.34	35.90	1.49
115	1.02	2.70	4.50	48.81	3.26
115	1.52	3.72	6.21	49.19	2.54
115	2.02	3.50	5.84	51.12	4.02
115	2.52	3.14	5.24	48.97	4.61
115	3.02	2.76	4.60	43.94	4.40
115	4.00	1.98	3.30	44.16	3.29
115	6.00	0.80	1.33	36.76	1.69
115	8.00	0.42	0.70	28.17	1.34
117	0.00	0.00	0.00	5.12	0.28
117	0.55	0.98	1.63	26.49	2.06
117	1.02	3.30	5.50	45.12	2.57
117	1.50	3.27	5.46	54.14	2.96
117	2.03	5.33	8.90	50.72	4.43
117	2.53	5.23	8.72	47.30	5.44
117	3.03	3.67	6.12	43.25	5.25
117	4.02	3.03	5.06	39.10	4.35
117	6.00	1.46	2.44	30.99	3.61
117	8.00	0.79	1.31	21.97	2.43
118	0.00	0.00	0.00	3.85	
118	0.50			14.80	
118	1.00	2.28	3.80	36.81	
118	1.50	3.82	6.37	43.60	
118	1.98	5.57	9.29	44.90	
118	2.50	4.62	7.70	39.21	
118	3.00	4.68	7.80	39.94	
118	4.00	3.59	5.99	34.41	
118	6.06	2.42	4.03	26.63	
118	8.00	1.49	2.49	20.40	
119	0.00	0.00	0.00	5.12	
119	0.50	1.26	2.11	41.20	
119	1.02	3.60	6.01	54.63	
119	1.50	3.16	5.27	59.70	
119	2.00	2.76	4.61	55.63	
119	2.50	2.32	3.87	49.27	
119	3.00	2.23	3.73	46.25	
119	4.00	1.53	2.55	42.67	
119	6.00	0.81	1.35	33.82	
119	8.00		0.37	23.97	
120	0.00	0.00	0.00	4.8	
120	0.52	3.64	6.10	52.52	
120	1.03	5.17	8.62	39.50	
120	1.50	6.21	10.36	49.37	
120	2.02	6.72	11.21	52.50	
120	2.52	4.29	0.50	51.07	
120	3.02	5.73	9.56	50.50	
120	4.03				
120	6.11				
120	8.00				
121	0.00	0.00	0.00	4.47	0.17
121	0.54	1.41	2.35	45.61	2.27
121	1.02	2.40	4.00	58.22	3.46
121	1.52	2.17	3.62	57.44	2.86
121	2.02	3.30	5.51	56.56	4.11
121	2.50	6.38	10.64	48.79	3.83
121	3.05	5.76	9.59	50.42	3.7
121	4.00	5.24	8.74	46.50	3.69
121	6.00	3.11	5.18	35.41	1.77
121	8.00	1.65	2.75	23.79	1.14

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
122	0.50	0.00	0.00	11.95	0.82
122	1.00	[illegible]	0.30	24.29	[illegible]
122	1.50	0.79	[illegible]	38.26	2.23
122	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
122	2.50	3.11	5.19	45.62	2.82
122	3.00	2.11	4.63	51.00	3.38
122	4.00	2.47	4.13	59.73	6.06
122	6.00	[illegible]	[illegible]	[illegible]	[illegible]
122	8.00	0.53	0.88	33.88	2.91
123	0.00	0.00	0.00	[illegible]	0.48
123	0.50	0.00	[illegible]	[illegible]	[illegible]
123	1.02	0.75	1.25	43.53	3.56
123	1.55	1.06	[illegible]	50.56	3.06
123	2.03	3.39	5.66	51.11	4.54
123	3.50	[illegible]	[illegible]	[illegible]	4.84
129	3.00	5.75	9.59	43.51	3.79
129	4.00	3.78	[illegible]	[illegible]	[illegible]
123	6.02	2.43	4.05	27.24	1.07
123	8.06	1.48	[illegible]	18.03	0.77
124	0.00	0.00	0.00	[illegible]	0.51
124	0.56	0.00	0.00	10.49	[illegible]
124	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
124	1.58	0.00	0.00	32.38	1.72
124	[illegible]	0.67	[illegible]	[illegible]	2.30
124	2.50	1.17	1.96	43.55	3.99
124	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
124	4.05	2.96	4.93	57.87	3.97
124	6.01	1.55	2.59	40.49	2.09
124	8.08	0.68	1.13	[illegible]	1.08
125	0.00	0.00	0.00	2.78	0.65
125	0.55	1.38	2.37	29.62	1.27
125	1.06	1.99	3.32	40.79	2.94
125	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
125	2.06	2.93	3.39	50.80	3.78
125	2.55	2.35	3.84	49.01	3.68
125	3.05	2.00	3.34	38.75	2.29
125	4.06	1.01	1.67	[illegible]	2.50
125	6.08	0.67	1.52	31.69	0.78
125	8.10	[illegible]	0.44	24.75	0.62
126	0.00	0.00	0.00	1.72	[illegible]
126	0.55	3.73	6.23	62.57	[illegible]
126	1.05	[illegible]	10.85	65.36	3.44
126	1.55	7.62	12.7	61.34	4.01
126	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
126	2.53	5.42	9.05	52.43	5.23
126	3.05	[illegible]	[illegible]	[illegible]	[illegible]
126	4.05	3.87	6.45	41.59	1.80
126	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
126	8.08	1.06	1.76	25.19	0.90
127	0.00	0.00	0.00	6.81	0.75
127	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
127	[illegible]	[illegible]	0.46	[illegible]	2.19
127	[illegible]	0.62	1.04	[illegible]	2.72
127	2.00	2.59	4.32	59.53	3.77
127	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
127	3.06	4.29	[illegible]	53.20	4.98
127	4.02	[illegible]	[illegible]	[illegible]	[illegible]
127	6.00	2.28	3.80	45.37	[illegible]
127	8.04	[illegible]	[illegible]	[illegible]	[illegible]
128	0.00	0.00	0.00	[illegible]	0.93
128	0.53	0.49	0.82	35.40	[illegible]
128	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
128	1.53	3.08	5.14	[illegible]	3.68
128	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
128	2.52	1.97	3.28	47.97	4.90
128	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
128	4.02	1.91	3.19	46.55	4.18
128	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
128	8.06	0.31	0.51	26.33	[illegible]

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
130	0.50	[illegible]	5.30	35.96	[illegible]
130	1.00	1.51	[illegible]	[illegible]	[illegible]
130	1.50	4.98	[illegible]	[illegible]	[illegible]
130	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
130	2.52	6.50	3.81	[illegible]	[illegible]
130	3.00	[illegible]	[illegible]	[illegible]	[illegible]
130	4.00	[illegible]	[illegible]	[illegible]	[illegible]
130	5.98	2.42	[illegible]	[illegible]	[illegible]
130	[illegible]	[illegible]	0.76	[illegible]	[illegible]
131	0.00	0.00	0.00	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131	1.00	[illegible]	[illegible]	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131	6.00	[illegible]	[illegible]	[illegible]	[illegible]
131	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	0.00	0.00	0.00	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	1.50	[illegible]	[illegible]	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	2.50	[illegible]	[illegible]	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	4.00	[illegible]	[illegible]	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
132	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	0.00	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	2.00	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
133	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
134

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
[illegible]	0.00	0.00	0.00	[illegible]	[illegible]
137	0.50		1.38	13.09	1.05
137	1.02	0.84	1.80	26.78	2.32
137	1.50	.34	1.78	31.5	2.26
137	2.00	1.37	2.35	34.26	1.72
137	2.52	4.93	3.76	36.64	6.02
137	2.98	4.56	2.13	33.21	5.15
137	4.00	3.07	1.90	34.16	2.82
137	6.00	.16	1.22	26.84	1.96
137	8.00	0.36	0.73	22.43	1.55
138	0.00	0.00	1.21	4.77	0.72
138	0.50		1.32	16.73	2.21
138	1.00	3.01	3.11	32.18	2.09
138	1.50	0.68	4.84	42.71	4.84
138	2.00	3.52	6.07	44.43	6.63
138	2.52	1.98	2.32	47.84	6.6
138	3.00	0.59	1.68	47.11	4.28
138	4.00	8.05	1.11	40.72	3.09
138	6.00	4.25	1.00	36.96	1.96
138	8.00	3.32	0.80	35.65	1.79
139	0.00	0.00	0.95	2.21	0.30
139	0.50	1.64	6.10	48.65	1.54
139	1.00	5.69	6.38	57.21	3.25
139	1.52	10.00	7.92	53.58	5.63
139	2.00	10.78	6.82	49.75	5.33
139	2.50	10.97	6.22	47.69	3.66
139	3.03	9.42	4.79	43.34	3.50
139	4.00	8.42	2.82	38.77	2.75
139	6.02	2.29	0.96	26.62	1.36
139	8.05	1.01	0.65	22.14	0.74
141	0.00	0.00	0.31	4.80	
141	0.61	1.11	3.19	20.04	
141	1.03	4.53	6.90	46.70	
141	1.56	4.57	6.72	52.19	
141	2.00	8.45	7.62	66.82	
141	2.50	8.07	5.42	52.88	
141	3.24	7.37	4.70	52.01	
141	4.03	5.57	3.72	55.01	
141	6.00	3.95	3.08	37.01	
141	8.02	1.34	1.52	25.04	
142	0.00	0.00		1.74	0.50
142	0.67	1.79	8.67	21.40	2.37
142	1.03	3.70	14.13	41.50	3.90
142	1.67	6.76	13.84	44.31	3.83
142	2.00	4.8	15.68	41.58	3.49
142	2.50	4.22	11.50	43.37	2.10
142	3.03	3.41	10.70	36.52	1.72
142	4.00	3.31	8.65	33.67	0.98
142	6.03	1.42	4.53	24.52	1.44
142	8.03	0.94	4.48	23.62	0.95
143	0.00	0.00	0.23	0.88	0.27
143	0.53		1.15	13.09	1.32
143	1.00		1.43	18.24	1.81
143	1.52		1.85	22.00	2.62
143	2.00		2.46	25.68	3.14
143	2.50	0.32	4.71	27.42	4.36
143	3.00		5.31	32.57	7.49
143	4.00	0.34	4.75	29.68	6.87
143	6.00	0.53	3.84	24.96	4.25
143	8.00	1.32	2.60	19.82	2.79
144	0.00	0.00	0.00	1.55	0.39
144	0.52	2.09	5.85	32.73	2.35
144	1.00	3.95	6.38	41.77	5.21
144	1.64	4.44	7.61	36.60	3.93
144	2.00	3.71	6.49	36.95	3.66
144	2.50	3.98	5.77	35.51	2.73
144	3.00	2.74	5.06	33.23	2.52
144	4.03	2.40	4.45	28.45	1.53
144	6.03	1.49	2.76	20.15	0.6
44	8.05	0.72	0.97	14.27	0.54

Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
145	0.00	0.00		[illegible]	[illegible]
145	0.50	0.00	2.39	30.59	1.51
145	1.00	1.21	5.21	55.52	3.30
145	1.53	1.99	4.28	63.50	3.64
145	2.00	2.96	4.45	62.07	5.20
145	2.50	4.13	3.32	54.59	5.03
145	2.98	4.35	3.13	51.28	4.75
145	4.00	3.04	3.35	50.54	2.50
145	6.00	1.56	2.31	44.87	1.75
145	8.05	0.76	1.60	37.73	1.56
46	0.00	0.00	0.23	10.53	0.33
146	0.50	0.20	2.24	25.41	0.84
146	1.03	0.42	3.50	53.22	2.05
146	1.53	2.80	3.91	60.66	1.95
146	2.05	6.39	4.23	68.05	2.70
46	2.53	7.72	3.9	70.82	2.11
146	3.02	8.70	3.28	71.67	4.06
46	4.00	6.53	3.54	72.70	2.81
146	6.02	4.07	2.71	53.06	1.64
146	8.05	2.56	1.91	36.95	1.21
147	0.00	0.00	0.25	15.38	1.58
147	0.63	2.99	4.85	98.75	1.51
147	1.05	5.13	5.75	43.90	1.50
147	1.58	6.55	6.94	82.34	2.04
147	2.16	6.30	6.32	96.57	1.65
147	2.58	5.40	4.63	69.38	2.55
147	3.10	5.26	4.90	77.24	2.57
147	4.08	4.18	3.97	70.11	3.69
147	6.03	2.76	2.34	52.69	2.16
147	8.02	1.93	1.98	49.66	1.87
148	0.00	0.00	0.00	3.37	0.74
148	0.60		2.33	24.90	1.71
148	1.05	4.46	8.07	62.27	4.34
148	1.60	3.99	7.14	62.89	3.21
148	2.16	5.22	5.95	60.17	7.36
148	2.66	5.30	5.11	55.01	8.13
148	3.08	5.35	4.93	53.21	6.04
148	4.10	4.15	3.67	47.30	4.57
148	6.02	2.73	2.90	38.70	1.95
148	8.02	1.90	2.00	28.86	1.74
149	0.00	0.00	0.00	3.91	0.40
149	0.53	0.00	0.32	11.82	0.96
149	1.03		2.29	29.26	1.89
149	1.53	0.49	3.28	32.08	2.92
149	2.00	0.99	3.75	38.87	3.97
149	2.52	2.61	3.76	38.17	4.70
149	3.03	3.28	3.94	45.55	5.08
149	4.06	2.76	3.51	46.16	5.36
149	6.00	1.65	1.42	32.77	2.46
149	7.98	0.92	1.01	32.46	1.79
150	0.00	0.00	0.00	5.22	0.62
150	0.49	2.22	5.36	40.28	1.76
150	1.00	3.60	4.91	52.89	3.65
150	1.50	8.41	4.26	56.17	5.60
150	2.00	7.13	3.52	49.70	4.40
150	2.52	5.89	3.32	48.92	4.54
150	3.00	5.50	2.98	34.48	3.47
150	4.00	3.75	2.63	25.46	2.85
150	6.06	1.20	1.56	25.06	1.55
150	8.19	0.40	0.94	21.09	1.16
151	0.00			4.41	0.46
151	0.52		5.08	36.73	1.31
151	1.00	5.21	7.04	45.19	5.20
151	1.50	7.24	5.29	41.48	2.93
151	2.02	7.69	6.75	42.01	3.70
151	2.50	5.46	5.14	39.03	3.35
151	3.00	5.30	5.24	36.27	2.67
151	4.00	3.34	4.78	30.91	1.77
151	6.06	1.49	2.54	26.39	1.47
151	8.19	0.53	1.81	22.94	0.96

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
150	0.00	0.00		0.79	1.31
152	0.50	3.5	5.22	30.77	2.85
152	1.00	5.84	4.58	35.16	6.77
152	1.50	5.39	4.35	39.63	7.56
152	2.00	5.67	4.97	44.02	5.1
152	2.50	5.52	3.95	42.16	3.77
152	3.00	4.43	1.85	36.36	2.13
152	4.00	3.40	1.87	34.50	2.13
152	6.00	1.65	0.75	24.36	1.46
152	8.10	0.6	1.27	20.48	1.16
153	0.00	0.00	0.00	0.00	1.51
153	0.50	0.00	0.00	16.33	1.56
153	1.00	0.00	0.00	23.66	2.06
153	1.50		1.25	30.08	1.83
153	2.05		2.29	35.17	1.87
153	2.52		3.26	38.90	2.46
153	3.00	2.51	4.78	41.85	3.33
153	4.00	4.80	2.55	36.14	6.45
153	6.27	3.40	1.00	20.74	3.92
153	8.22	2.14	1.55	13.82	2.76
154	0.00	0.00	0.00	3.75	0.73
154	0.56	2.60	4.42	32.56	2.21
154	1.00	6.23	3.20	39.91	3.20
154	1.50	8.73	2.70	46.80	3.24
154	2.02	10.09	1.98	53.42	3.35
154	2.53	11.30	0.00	25.70	4.96
154	3.00	6.50	0.00	22.73	5.42
154	4.00	4.33	0.00	20.40	4.10
154	6.03	0.75	0.00	15.70	1.74
154	8.13		0.00	11.85	1.23
155	0.00	0.00	0.00	2.01	0.57
155	0.50	0.38	2.01	16.61	1.53
155	1.00	1.56	2.30	29.70	2.02
155	1.50	4.02	2.27	40.37	1.97
155	2.00	11.11	3.16	49.68	2.27
155	2.53	10.61	3.51	48.33	4.10
155	3.00	5.50	2.53	42.29	6.29
155	4.00	6.30	1.46	31.40	3.60
155	6.05	4.09	1.35	28.09	2.00
155	8.05	1.64	0.74	9.35	1.4
156	0.00	0.00	0.00	2.63	0.28
156	0.50	0.00	2.68	36.65	0.75
156	1.00	0.30	3.09	45.44	0.99
156	1.50	2.93	3.91	4.35	0.96
156	2.05	4.57	3.55	50.09	1.75
156	2.50	6.93	4.15	39.03	1.83
156	3.00	9.35	2.12	34.26	2.37
156	4.00	5.06	2.05	29.90	0.93
156	6.10	2.98	1.64	21.26	1.60
156	8.27	1.20	1.40	16.74	1.11
158	0.00	0.00	0.00	1.34	0.53
158	0.50	1.77	5.15	20.12	1.48
158	1.00	5.70	3.08	6.74	3.08
158	1.50	5.49	3.29	36.53	3.5
158	2.00	5.55	3.78	36.39	5.22
158	2.50	5.13	2.61	37.22	3.16
158	2.97	4.77	1.96	31.15	4.71
158	4.00	2.79	1.30	27.49	2.72
158	6.02	0.65	0.56	18.85	1.25
158	8.06		0.00	13.44	0.97
159	0.00	0.00	0.00	3.10	0.37
159	0.50		1.05	31.49	0.71
159	1.00		2.35	60.19	1.20
159	1.48	0.51	4.03	66.78	1.62
159	2.02	2.06	4.65	69.39	1.58
159	2.50	5.49	4.75	73.60	1.38
159	2.97	8.03	5.44	69.01	1.59
159	4.02	8.23	4.35	59.72	3.71
159	6.02	4.89	2.51	50.93	
159	8.01	2.78	2.02	44.77	1.75

Sub-ject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
160	0.00	0.00	0.00	22.45	1.08
160	0.55		1.08	31.58	1.69
160	1.02	0.66	1.23	44.45	3.01
160	1.50	1.40	1.32	53.16	3.35
160	2.00	1.85	2.40	60.82	4.52
160	2.50	2.03	1.31	65.37	5.08
160	3.00	3.05	3.52	68.82	6.36
160	4.00	1.33	3.40	65.47	7.26
160	6.00	0.65	0.54	26.12	4.43
160	8.00		0.00	2.85	3.35
161	0.00	0.50	0.00	3.79	0.30
161	0.50	2.28	0.54	56.32	2.15
161	1.00	5.26	3.04	52.47	3.74
161	1.52	5.71	3.90	65.32	3.25
161	2.02	6.15	3.09	44.78	3.04
161	2.50	6.29	3.32	42.32	4.03
161	3.02	5.46	2.46	39.20	2.11
161	4.02	3.11	.44	32.19	1.71
161	6.02	1.02	1.00	25.74	1.19
161	8.02		0.73	18.78	0.75
162	0.00	0.00	0.00	3.08	0.66
162	0.50	0.51	3.49	34.02	1.91
162	1.02	5.21	5.78	49.77	3.41
162	1.52	7.00	5.34	52.58	4.62
162	2.02	7.85	4.43	49.56	8.30
162	2.52	8.31	4.64	49.8	5.40
162	3.02	6.98	4.27	3.16	3.43
162	4.02	4.17	3.01	35.96	2.92
162	6.05	1.24	.37	24.6	1.50
162	8.02	3.94	1.06	21.47	1.02
163	0.00	0.00	0.00	0.00	0.00
163	0.50		1.89	27.77	0.70
163	1.00	2.17	4.00	44.51	2.06
163	1.50	4.02	3.72	57.55	3.70
163	2.00	4.29	4.93	56.19	4.43
163	2.50	6.97	4.78	61.66	4.01
163	3.00	6.05	4.07	50.74	5.03
163	4.00	5.83	3.47	36.27	3.43
163	6.00	3.91	2.39	41.09	1.61
163	8.00	2.87	1.43	32.67	0.81
164	0.00		0.00	7.77	0.93
164	0.53		6.58	51.10	1.82
164	1.03	7.15	4.18	47.40	3.27
164	1.52	8.33	3.32	58.38	4.55
164	2.02	8.70	2.43	58.76	5.26
164	2.53	5.29	1.99	51.30	4.95
164	3.03	7.68	1.66	52.47	3.85
164	4.03	6.71	1.13	43.47	2.81
164	6.13	3.69	0.63	28.6	1.52
164	8.03	1.95		18.58	
165	0.00	0.00	0.00	6.79	0.32
165	0.50		2.69	29.92	2.11
165	1.00		3.25	40.24	2.55
165	1.50	0.69	4.24	47.36	4.08
165	2.00	3.92	4.96	42.63	7.22
165	2.50	5.17	4.24	39.21	7.01
165	3.00	2.97	3.07	39.70	4.53
165	4.00	1.91	2.56	36.63	2.56
165	6.00	0.61	1.51	29.76	1.34
165	8.00		1.36	25.55	1.01
166	0.00	0.00	0.00	17.11	0.37
166	0.50		0.00	36.11	1.78
166	1.00	0.45	2.29	57.16	2.83
166	1.50	2.41	3.34	61.41	3.09
166	2.02	3.48	3.61	64.61	2.84
166	2.50	3.96	2.77	60.70	4.13
166	3.02	2.60	2.55	59.92	6.65
166	4.03	1.82	1.95	63.86	4.37
166	6.03	0.41	1.20	50.75	2.60
166	8.02		0.80	44.03	2.15

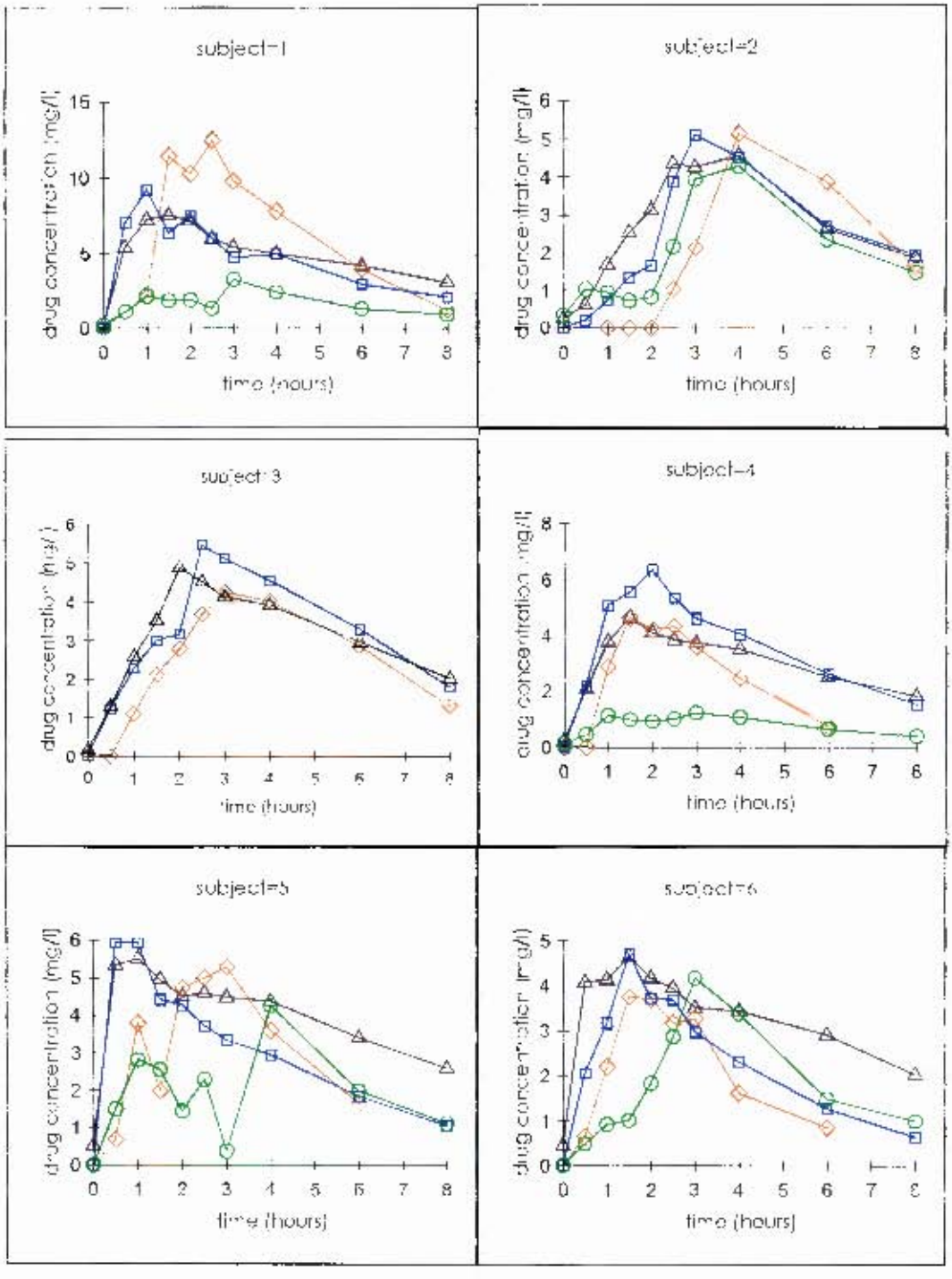
Subject	time	[R] (mg/l)	[H] (mg/l)	[Z] (mg/l)	[E] (mg/l)
167	0.00	0.00	0.00	3.34	0.56
167	0.50	0.00	0.56	20.36	0.94
167	1.00		0.50	31.94	1.33
167	1.50		3.11	37.26	1.63
167	2.00		3.00	42.22	2.56
167	2.52	1.14	1.70	41.99	3.95
167	3.03	1.65	1.52	38.80	6.03
167	4.02	1.92	0.70	31.20	4.15
167	6.03	2.27		24.41	1.61
167	8.02	1.22	0.00	18.52	0.55
168	0.00		0.00	6.85	0.18
168	0.50		2.83	25.41	1.29
168	1.00		2.86	31.30	1.85
168	1.50	1.25	4.52	43.09	2.58
168	2.00	3.64	3.43	51.44	2.70
168	2.50	3.73	2.95	40.68	2.32
168	3.00	3.70	2.40	34.35	8.08
168	4.00	2.29	1.84	32.48	3.74
168	6.00	1.51	1.29	27.07	2.23
168	8.00		0.71	20.60	1.64
169	0.00		0.00	9.54	0.98
169	0.50	1.35	3.72	33.26	1.38
169	1.00	6.02	4.03	48.81	3.33
169	1.50	5.40	2.74	49.34	5.05
169	2.00	5.11	3.32	50.84	6.72
169	2.50	9.27	2.64	52.59	7.94
169	3.00	8.54	1.57	32.50	9.53
169	4.00	5.92	1.51	46.59	6.26
169	6.00	3.85	0.92	40.25	2.31
169	8.00	2.56		30.67	1.46

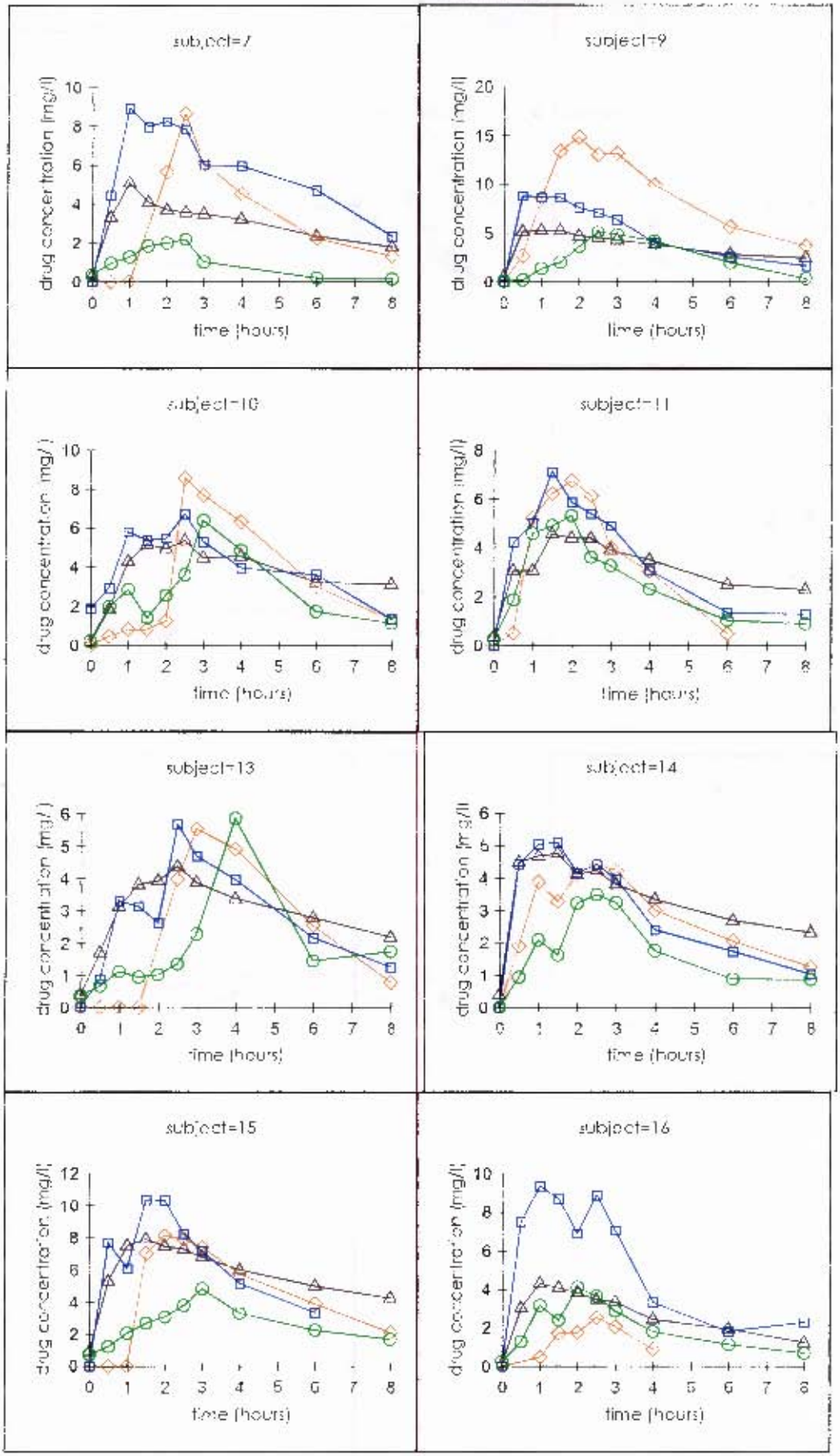
[P] : Pamaldin concentration
[H]: isoniazid concentration
[Z]: pyrazinamide concentration
[E]: ethambutol concentration

DRUG CONCENTRATION-TIME PROFILES FOR EACH SUBJECT

Concentration-time profiles of rifampicin, isoniazid, pyrazinamide and ethambutol in 142 subjects.

Rifampicin
Isoniazid
Pyrazinamide concentration divided by a factor of 10
Ethambutol

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time (hours)
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drug concentration (mg/l)
time (hours)
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time (hours)
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drug concentration (mg/l)
time (hours)
subject=13
drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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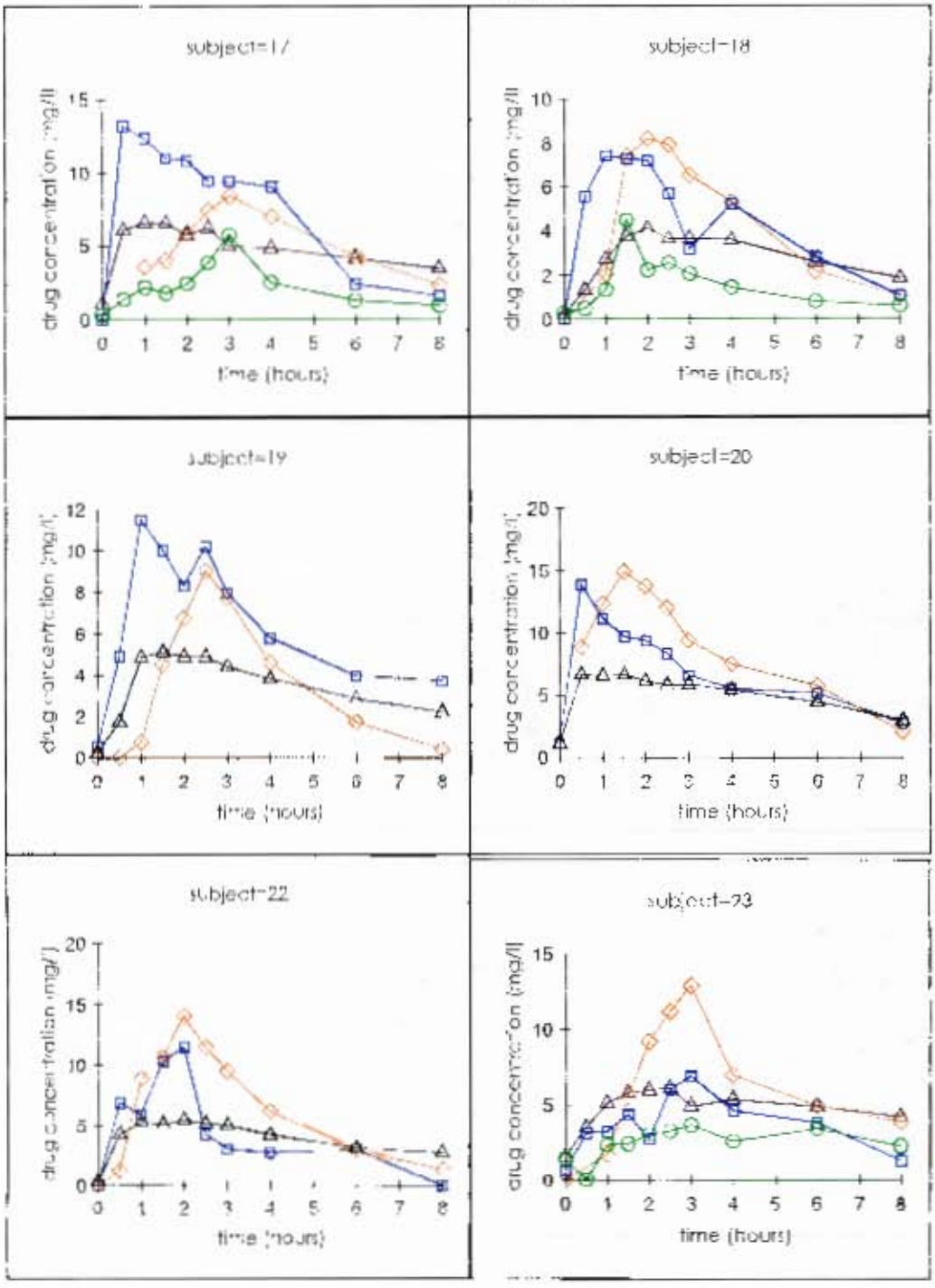
drug concentration (mg/l)
time (hours)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time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
subject=22
drug concentration (mg/l)
time (hours)
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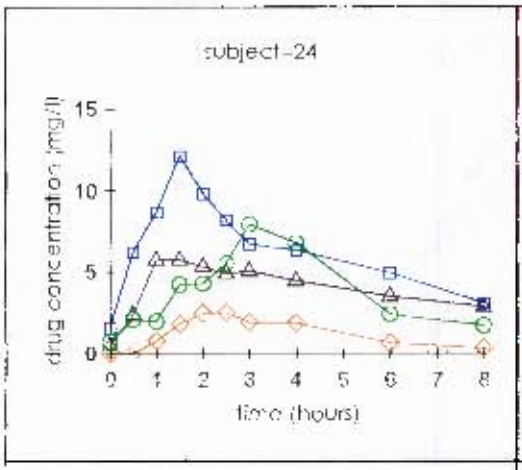
drug concentration (mg/l)
time (hours)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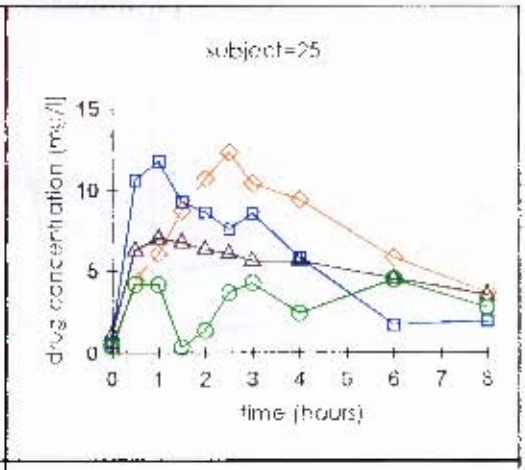
time (hours)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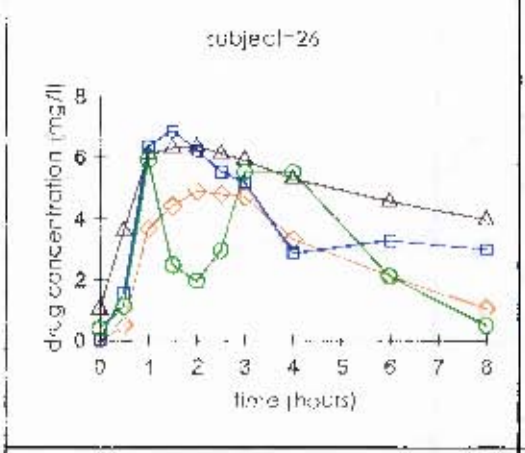
drug concentration (mg/l)
time (hours)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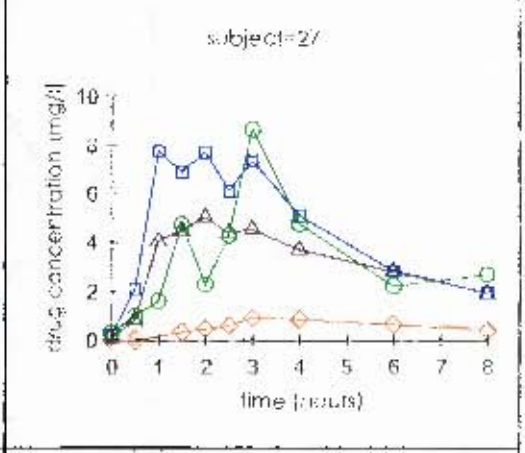
time (hours)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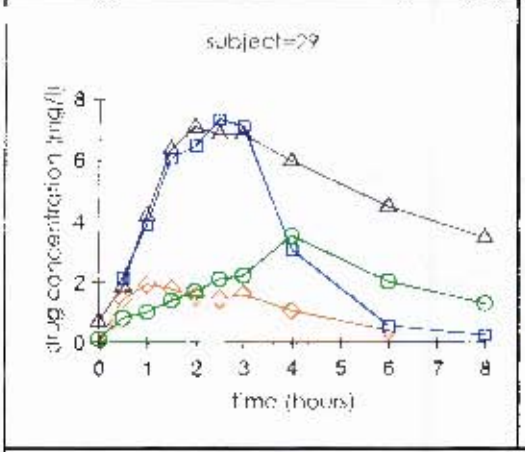
drug concentration (mg/l)
time (hours)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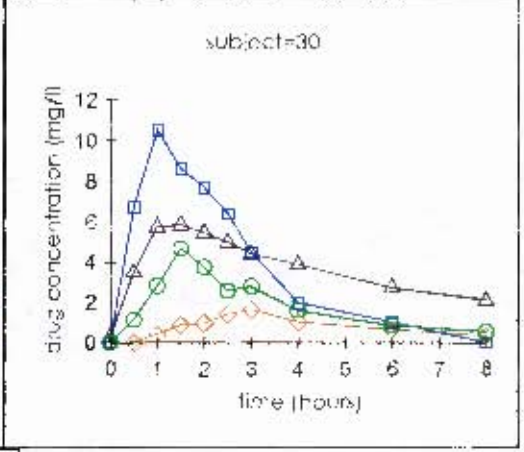
time (hours)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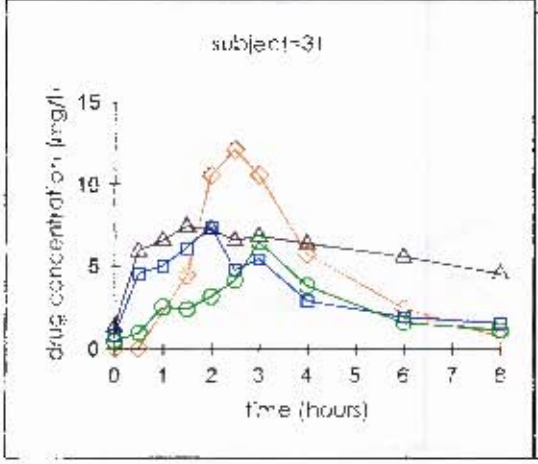
time (hours)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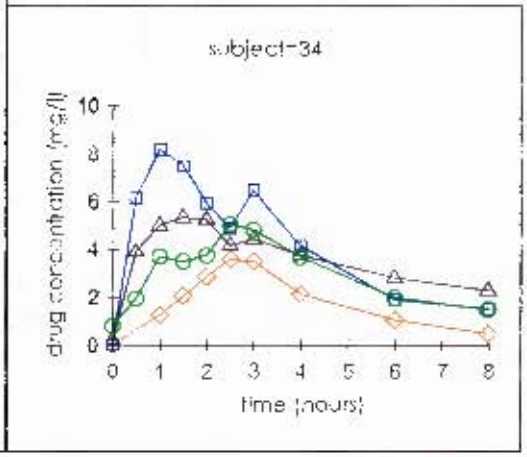
time (hours)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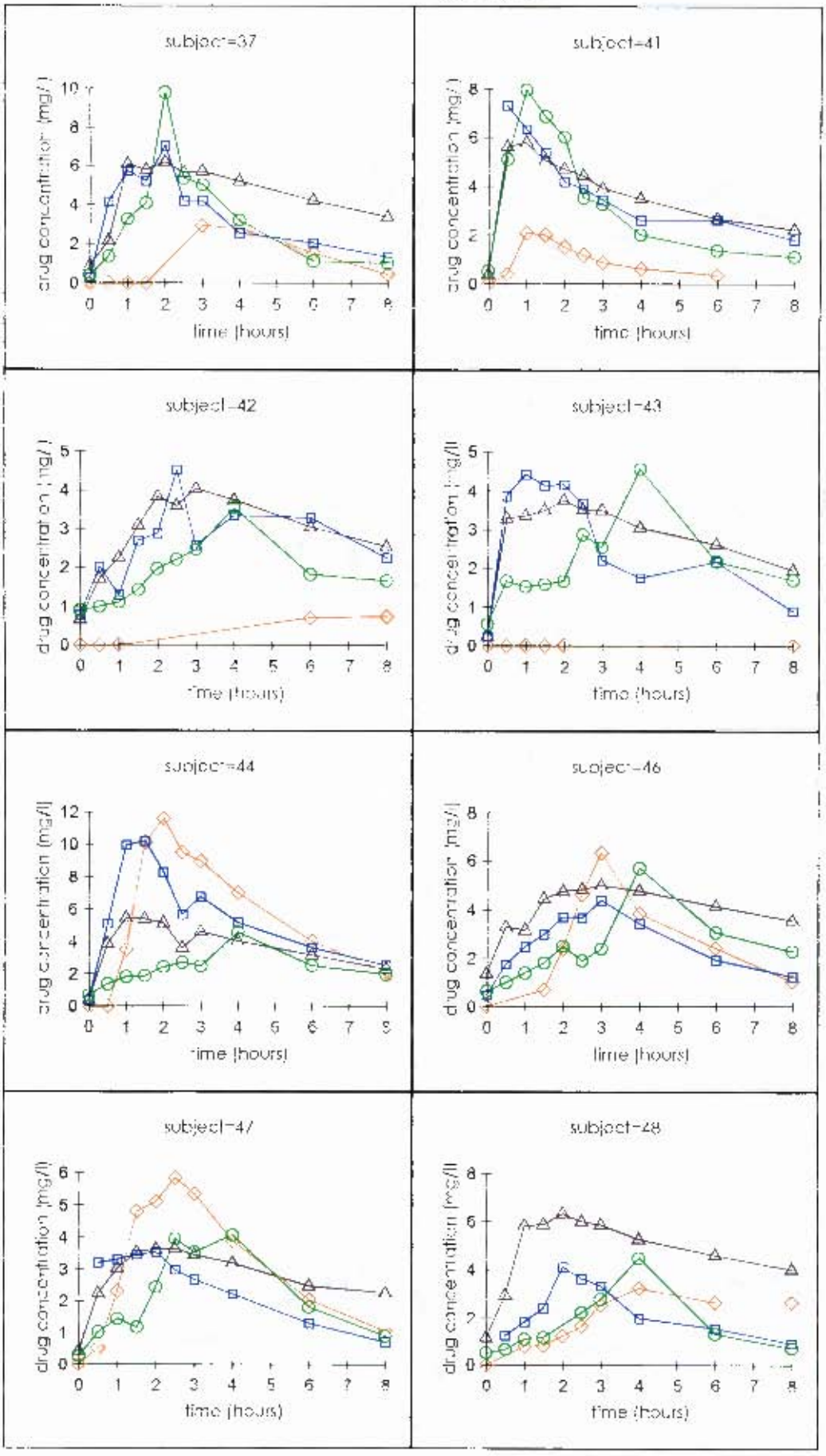
time (hours)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time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
subject=44
drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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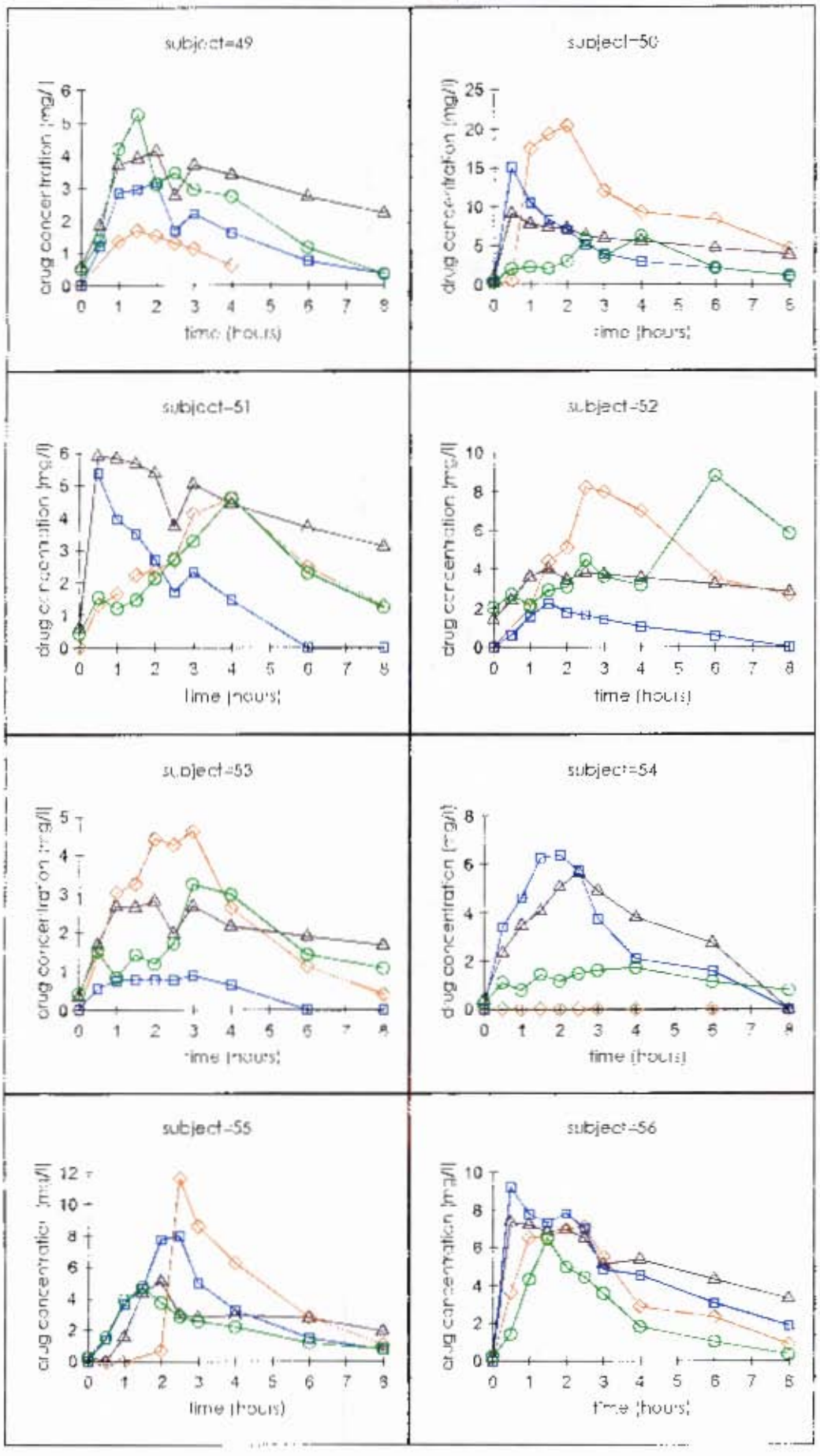
drug concentration (mg/l)
time (hours)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time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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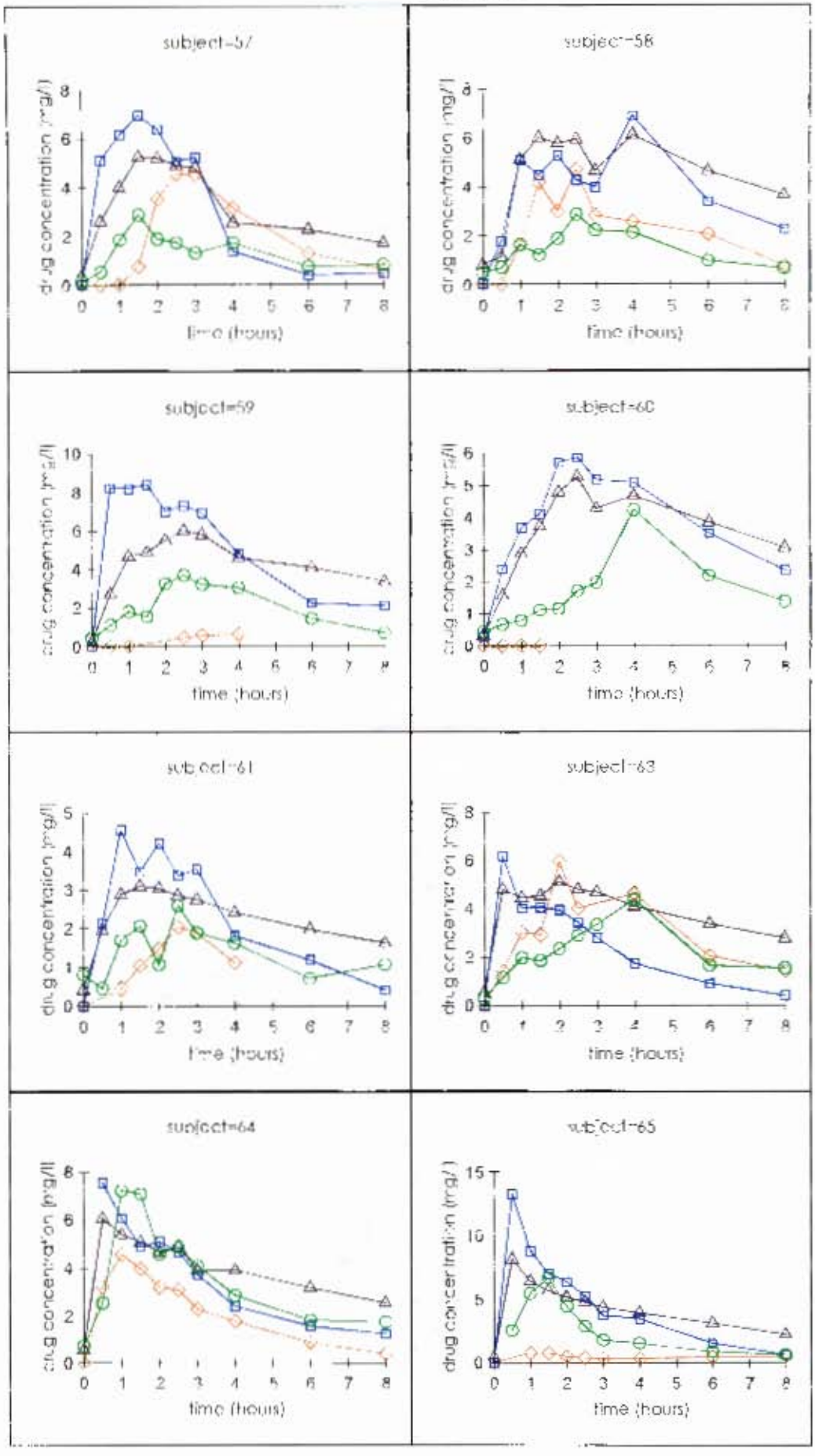
drug concentration (mg/l)
time (hours)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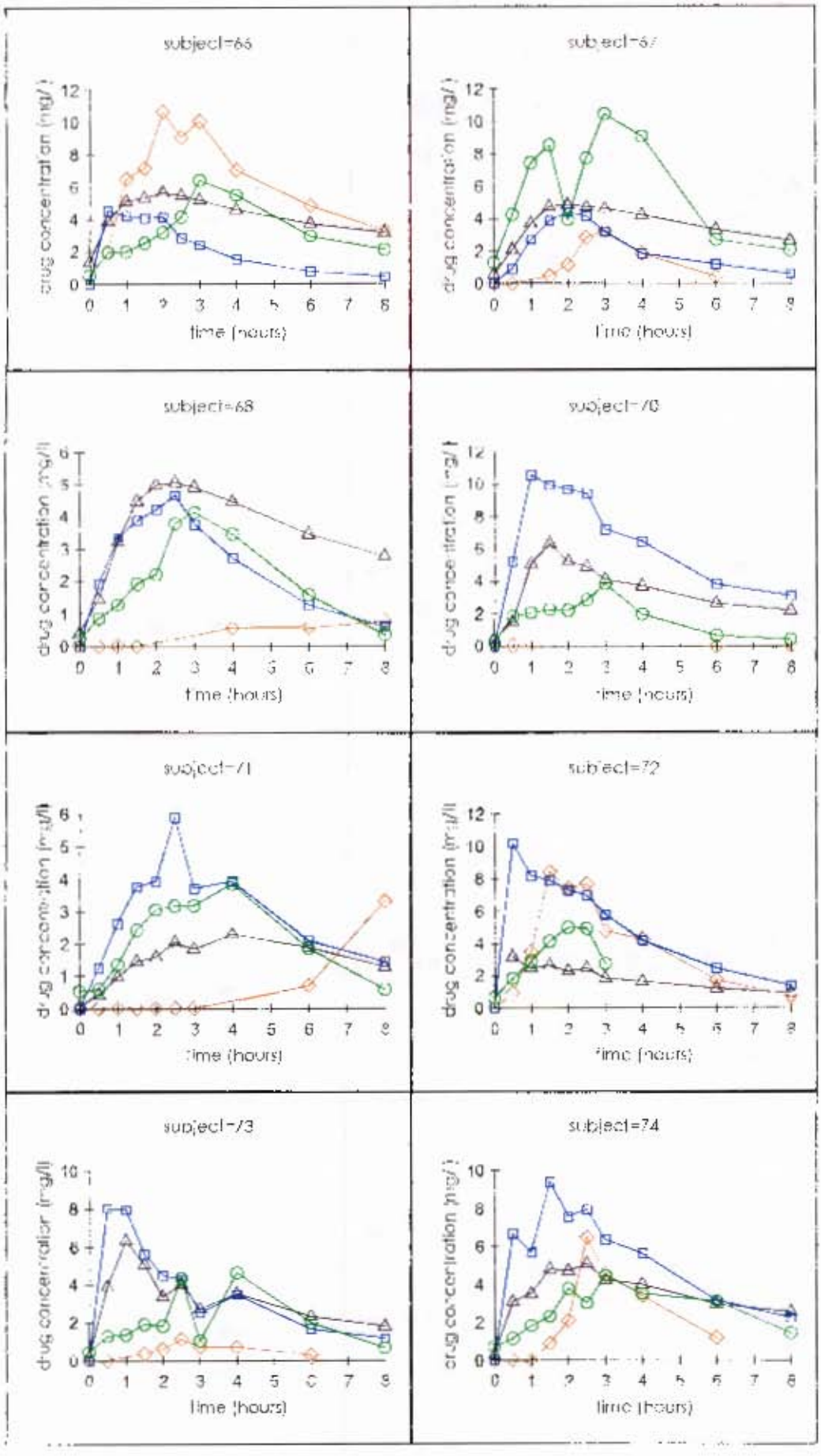
time (hours)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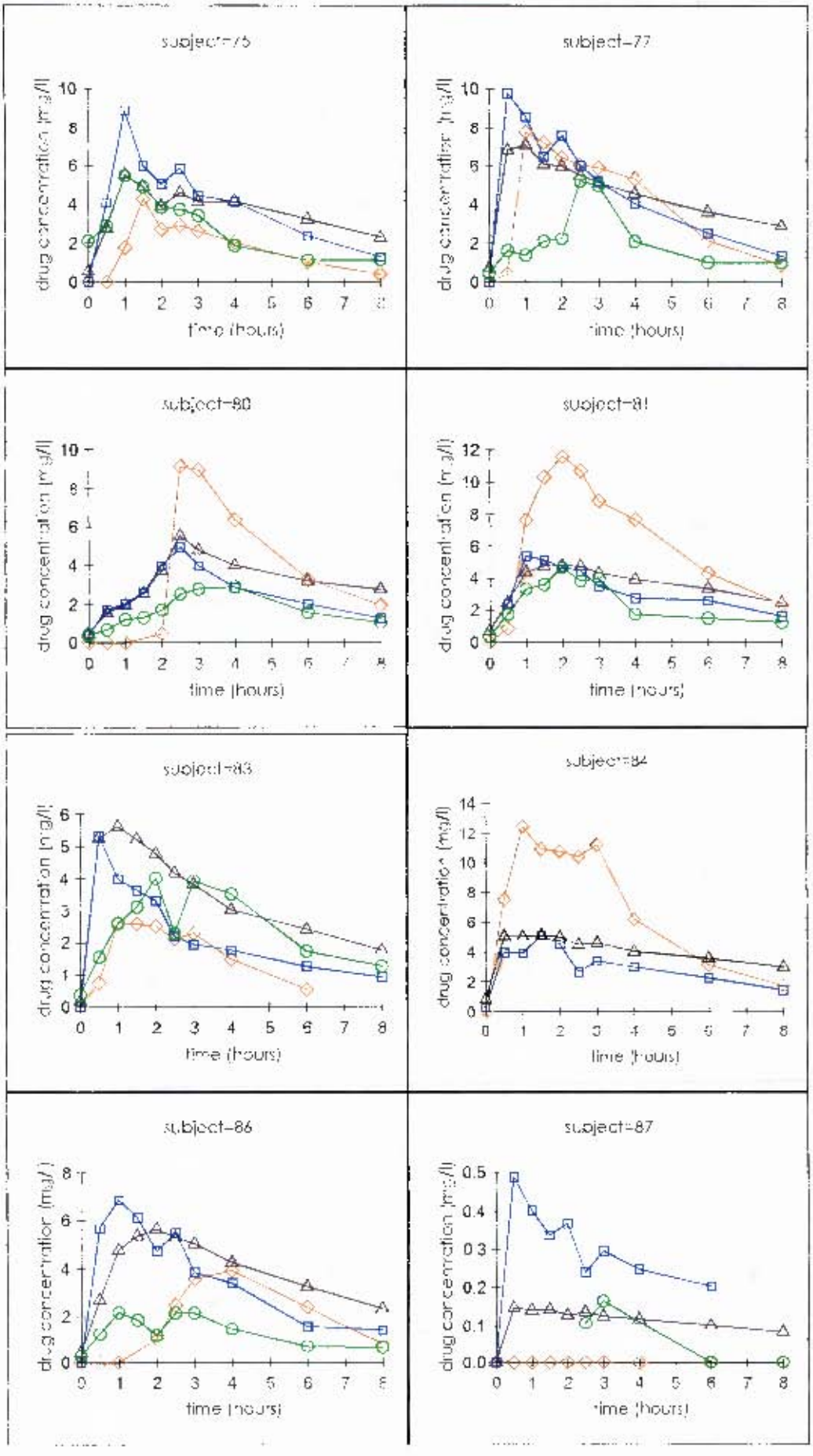
drug concentration (mg/l)
time (hours)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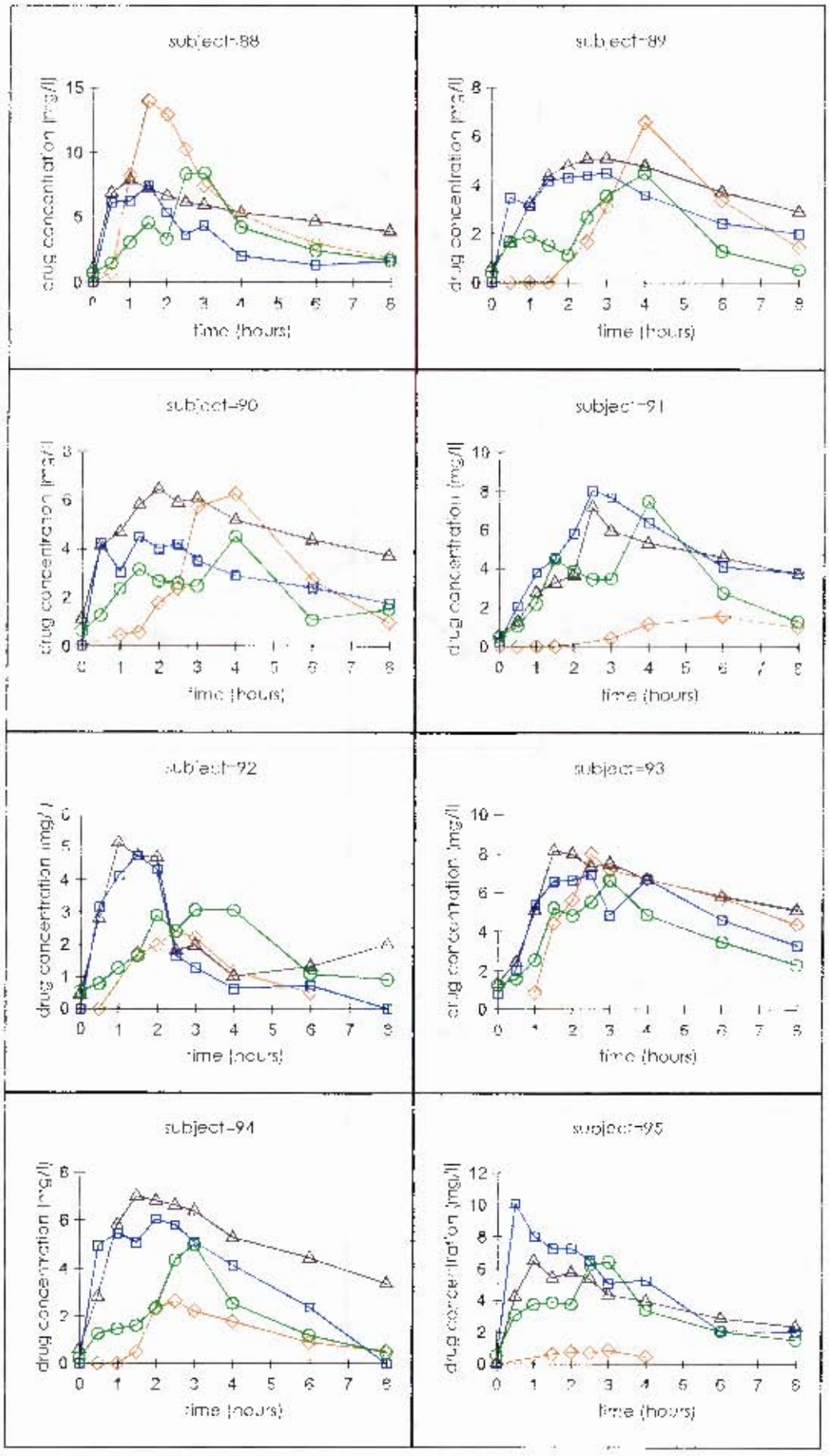
drug concentration (mg/l)
time (hours)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time (hours)
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time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
subject=92
drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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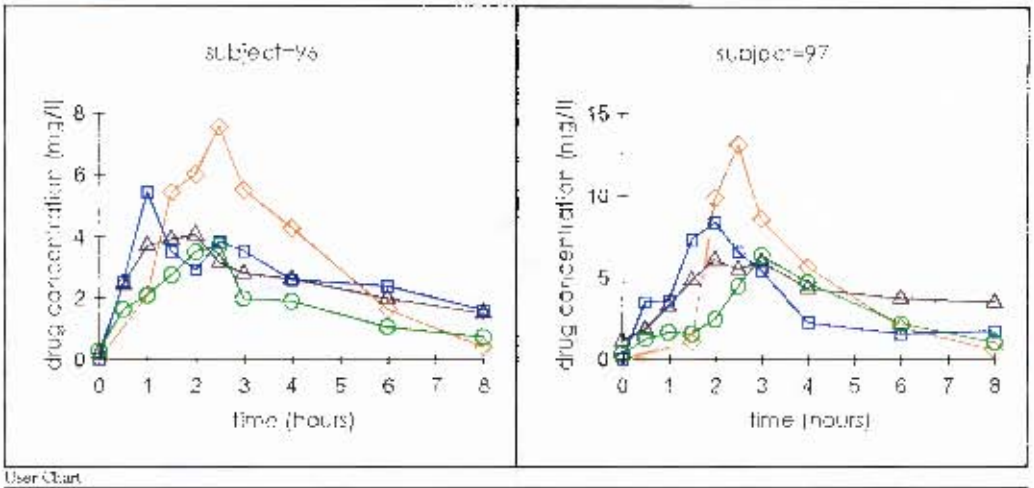
drug concentration (mg/l)
time (hours)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drug concentration (mg/l)
time (hours)

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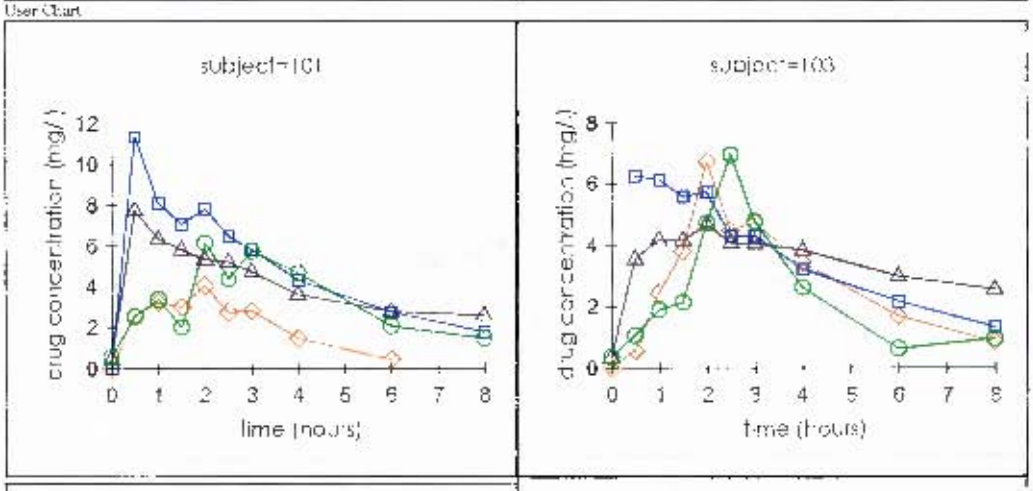
drug concentration (mg/l)
time (hours)

User Chart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time (hours)

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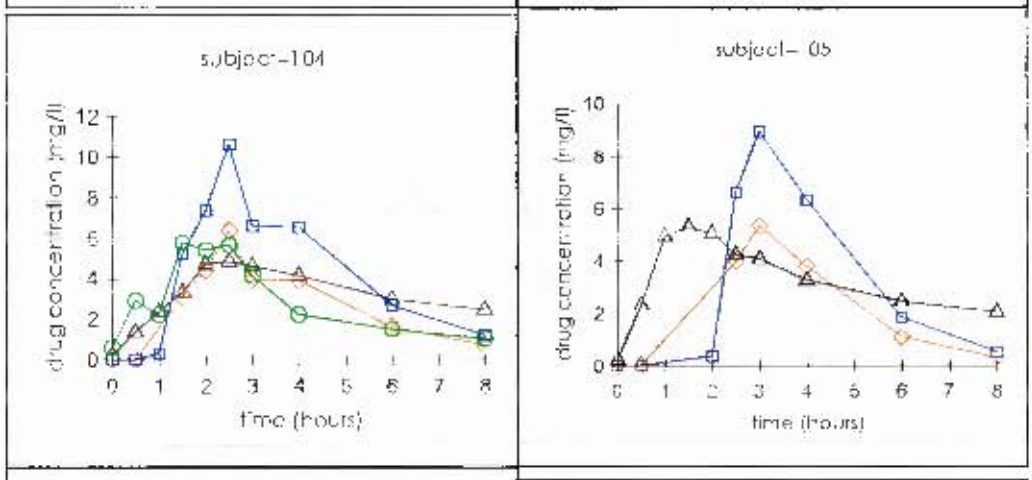
drug concentration (mg/l)
time (hours)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time (hours)

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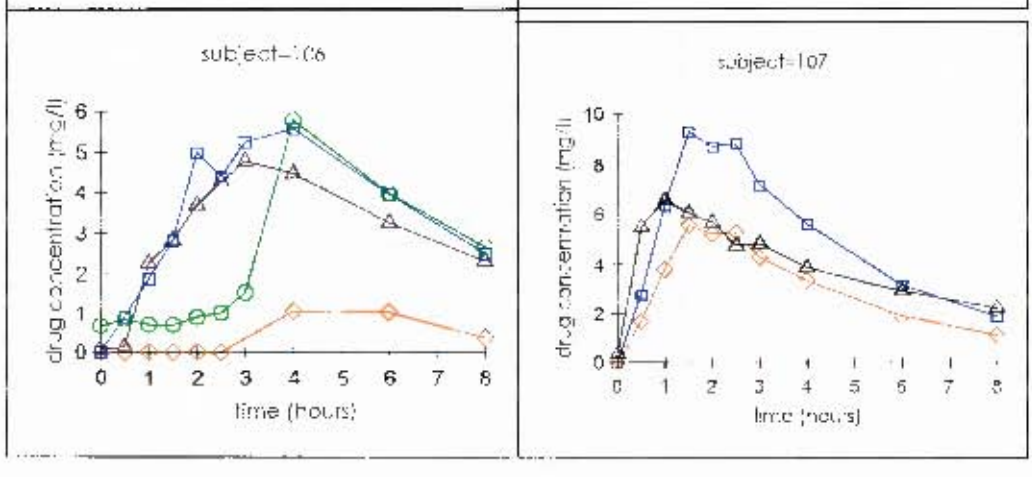
drug concentration (mg/l)
time (hours)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time (hours)

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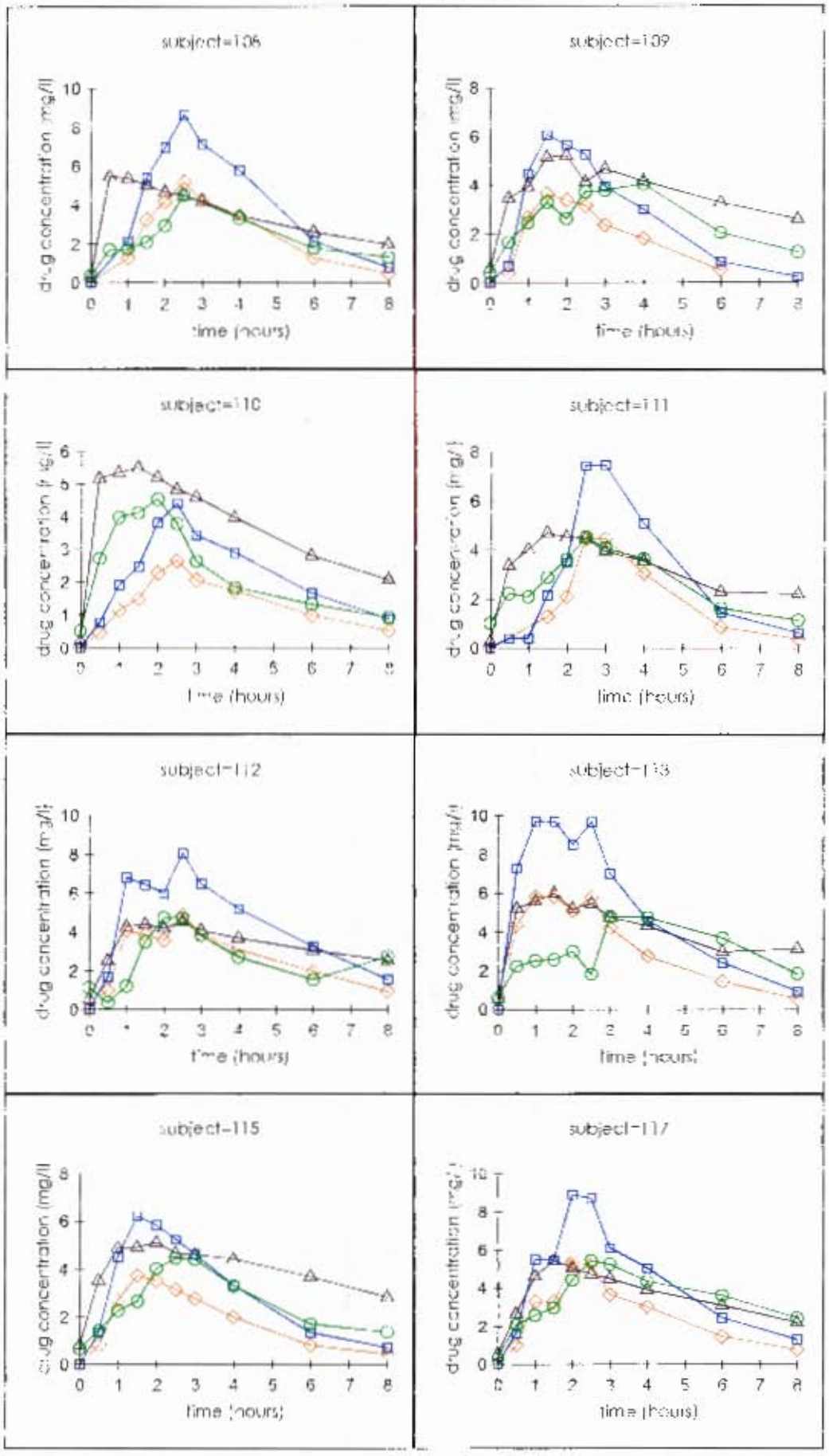
drug concentration (mg/l)
time (hours)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subject=112
subject=113
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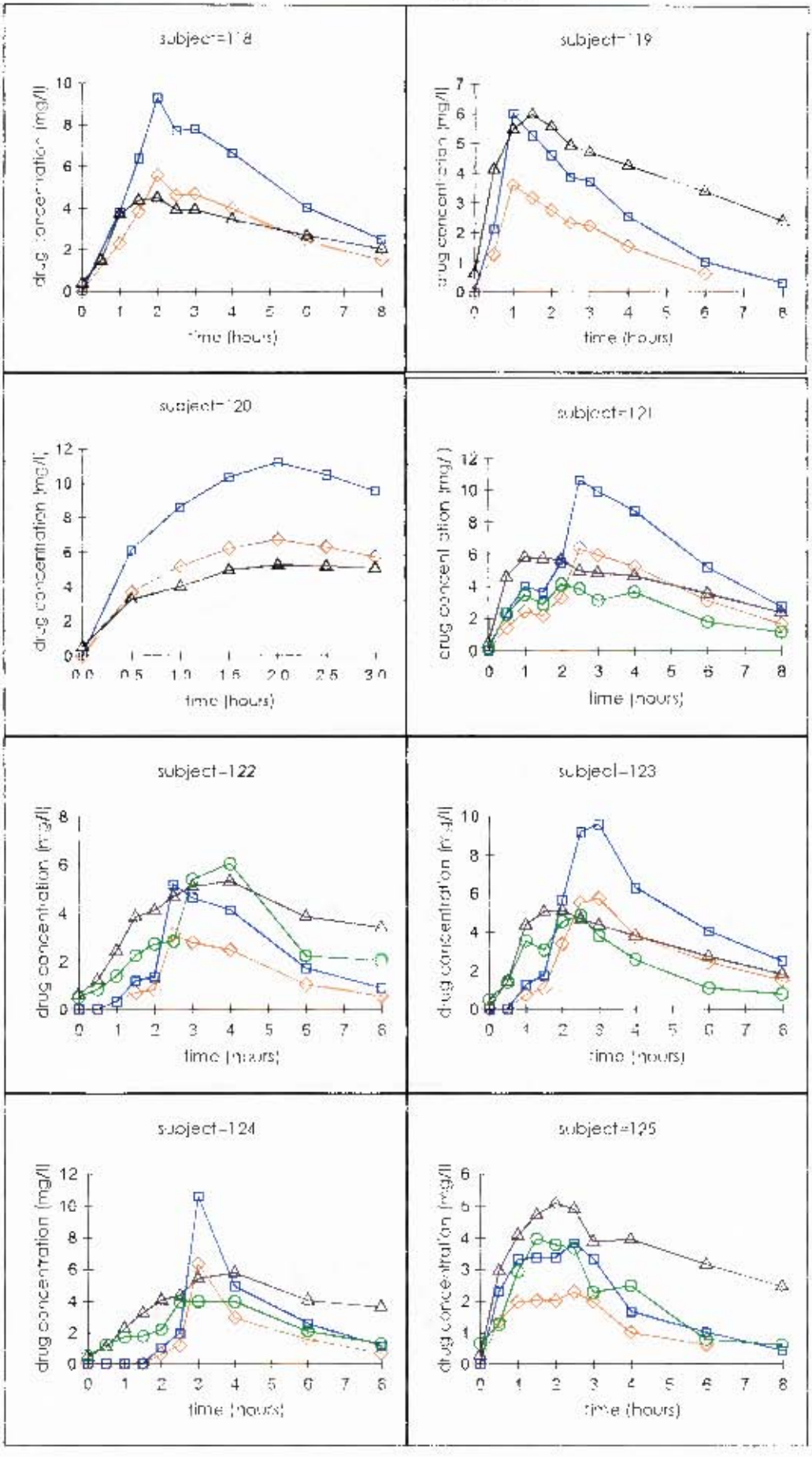
subject=117
drug concentration (mg/l)
time (hours)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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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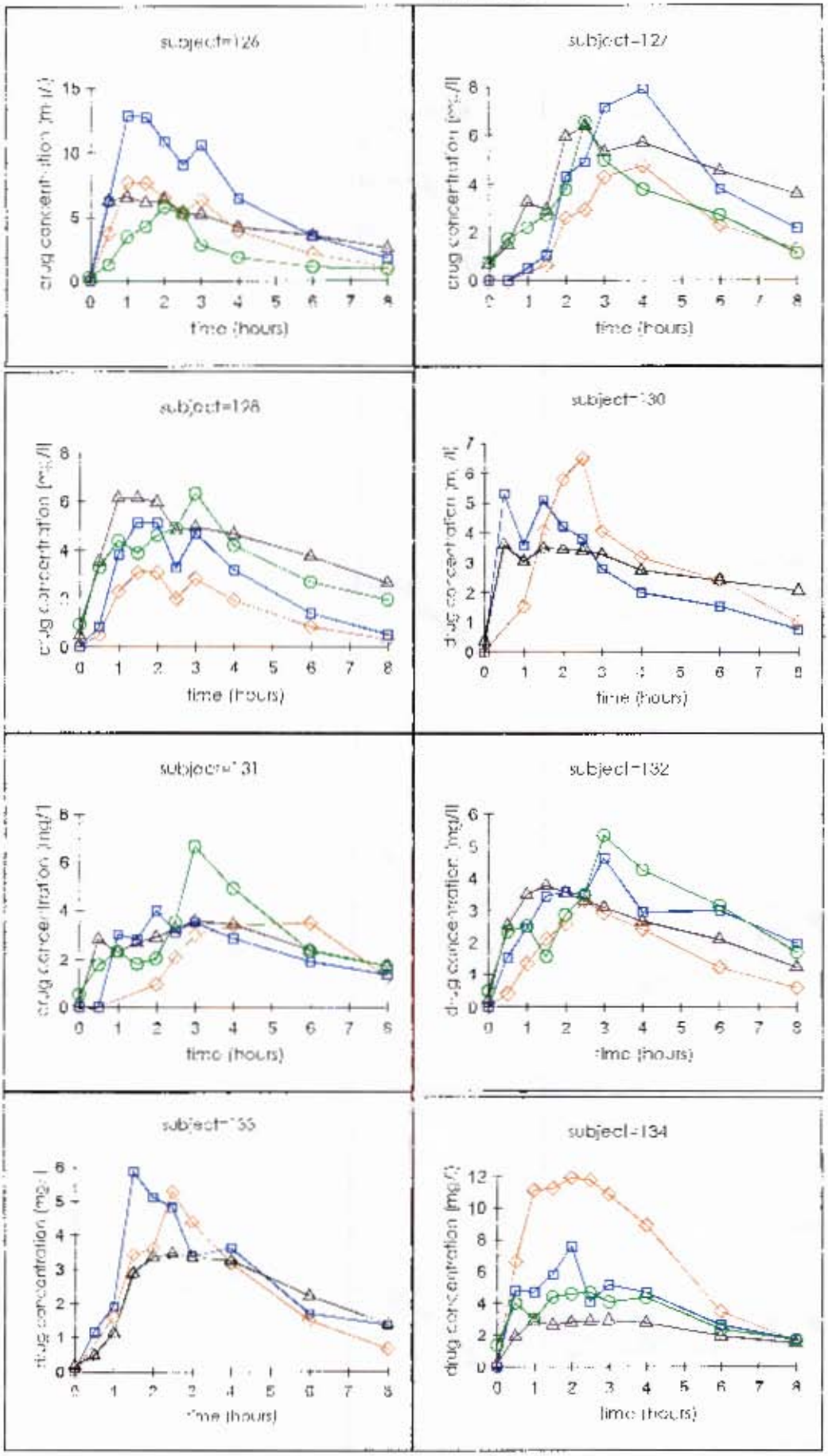
drug concentration (mg/l)
time (hours)
subject=121
drug concentration (mg/l)
time (hours)
subject=122
drug concentration (mg/l)
time (hours)
subject=123
drug concentration (mg/l)
time (hours)
subject=124
drug concentration (mg/l)
time (hours)
subject=125
drug concentration (mg/l)
time (hours)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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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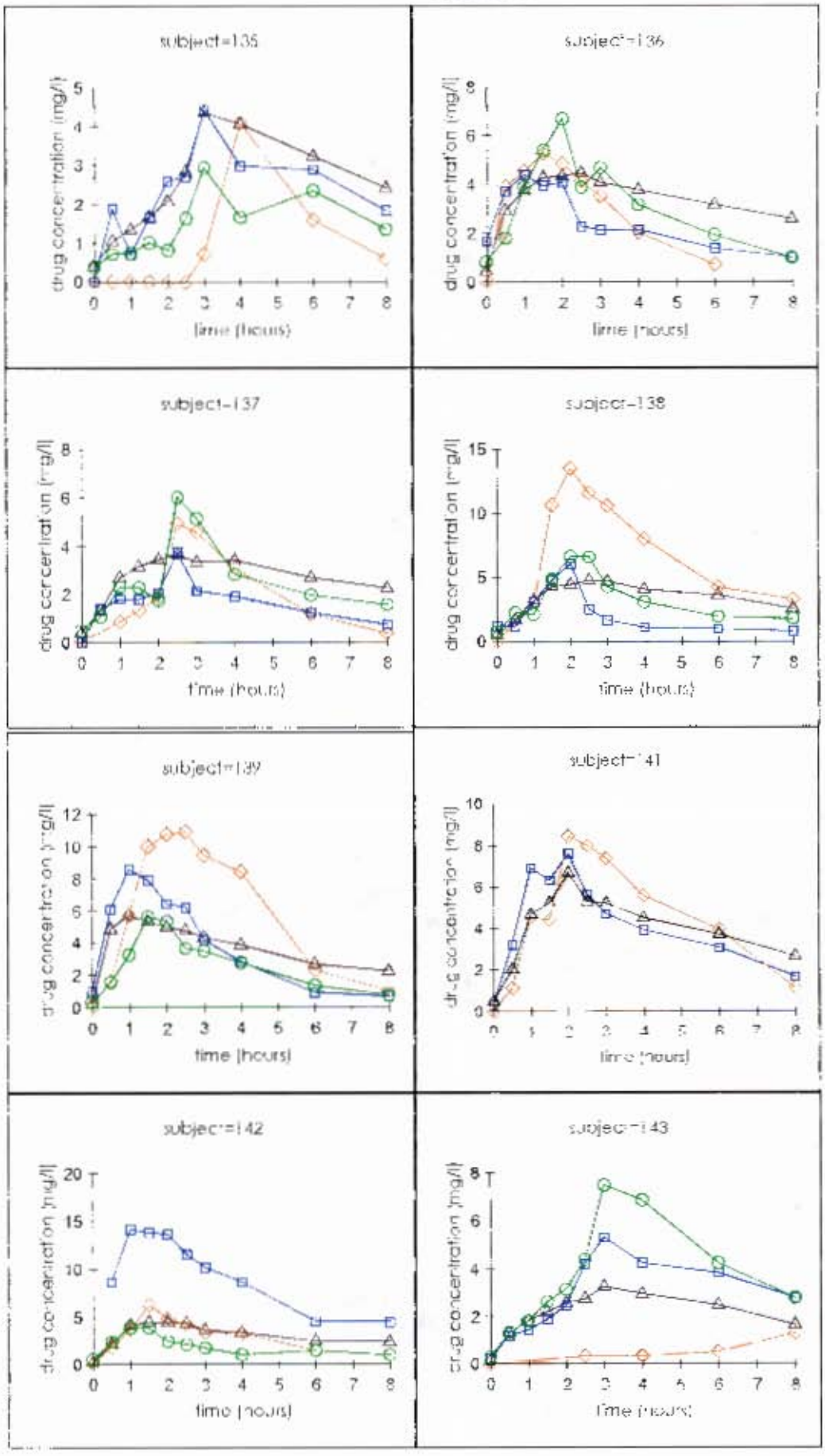
drug concentration (mg/l)
time (hours)
subject=130
drug concentration (mg/l)
time (hours)
subject=131
drug concentration (mg/l)
time (hours)
subject=132
drug concentration (mg/l)
time (hours)
subject=133
drug concentration (mg/l)
time (hours)
subject=134
drug concentration (mg/l)
time (hours)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drug concentration (mg/l)
time (hours)
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drug concentration (mg/l)
time (hours)
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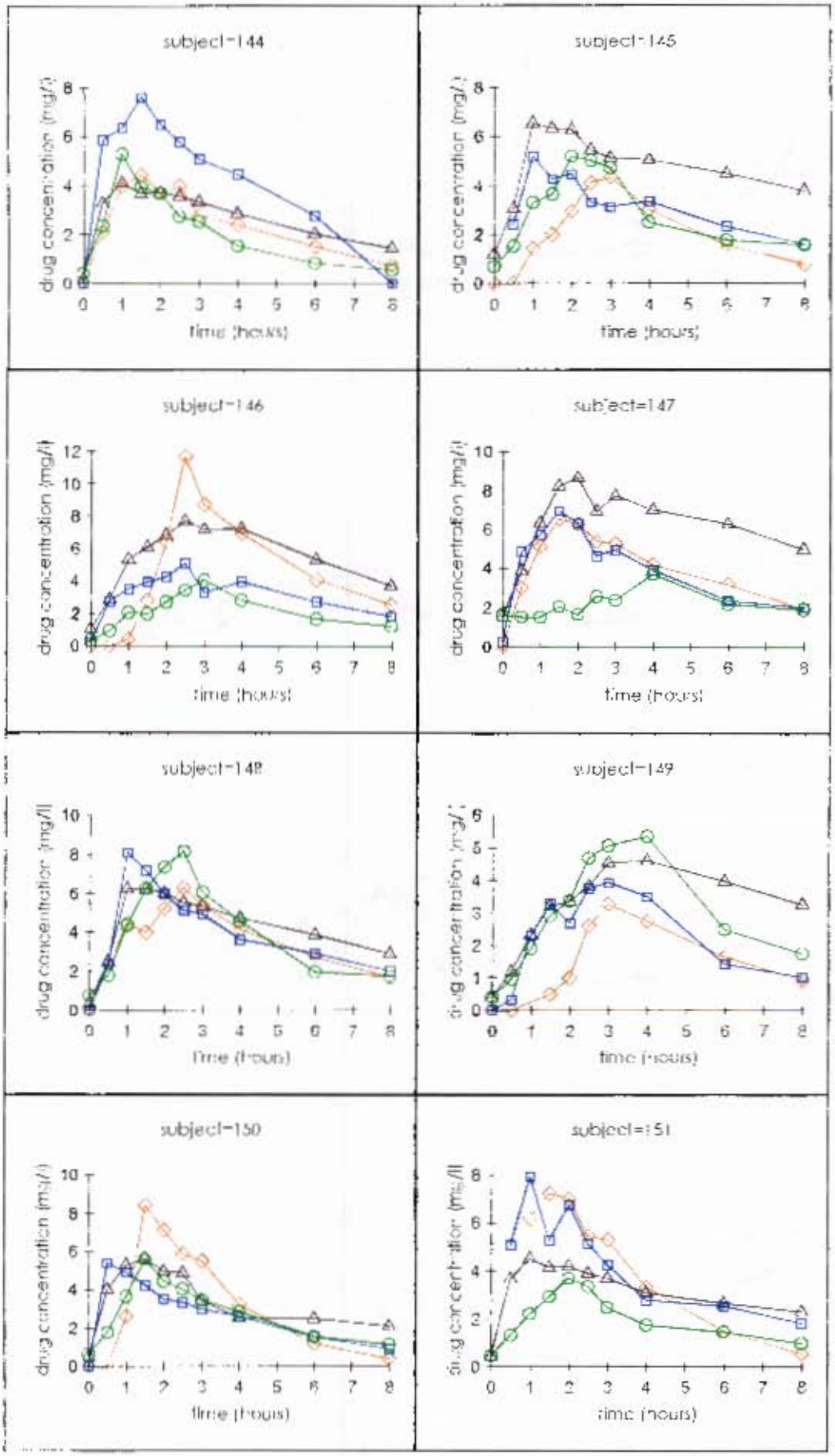
drug concentration (mg/l)
time (hours)
subject=138
drug concentration (mg/l)
time (hours)
subject=139
drug concentration (mg/l)
time (hours)
subject=141
drug concentration (mg/l)
time (hours)
subject=142
drug concentration (mg/l)
time (hours)
subject=143
drug concentration (mg/l)
time (hours)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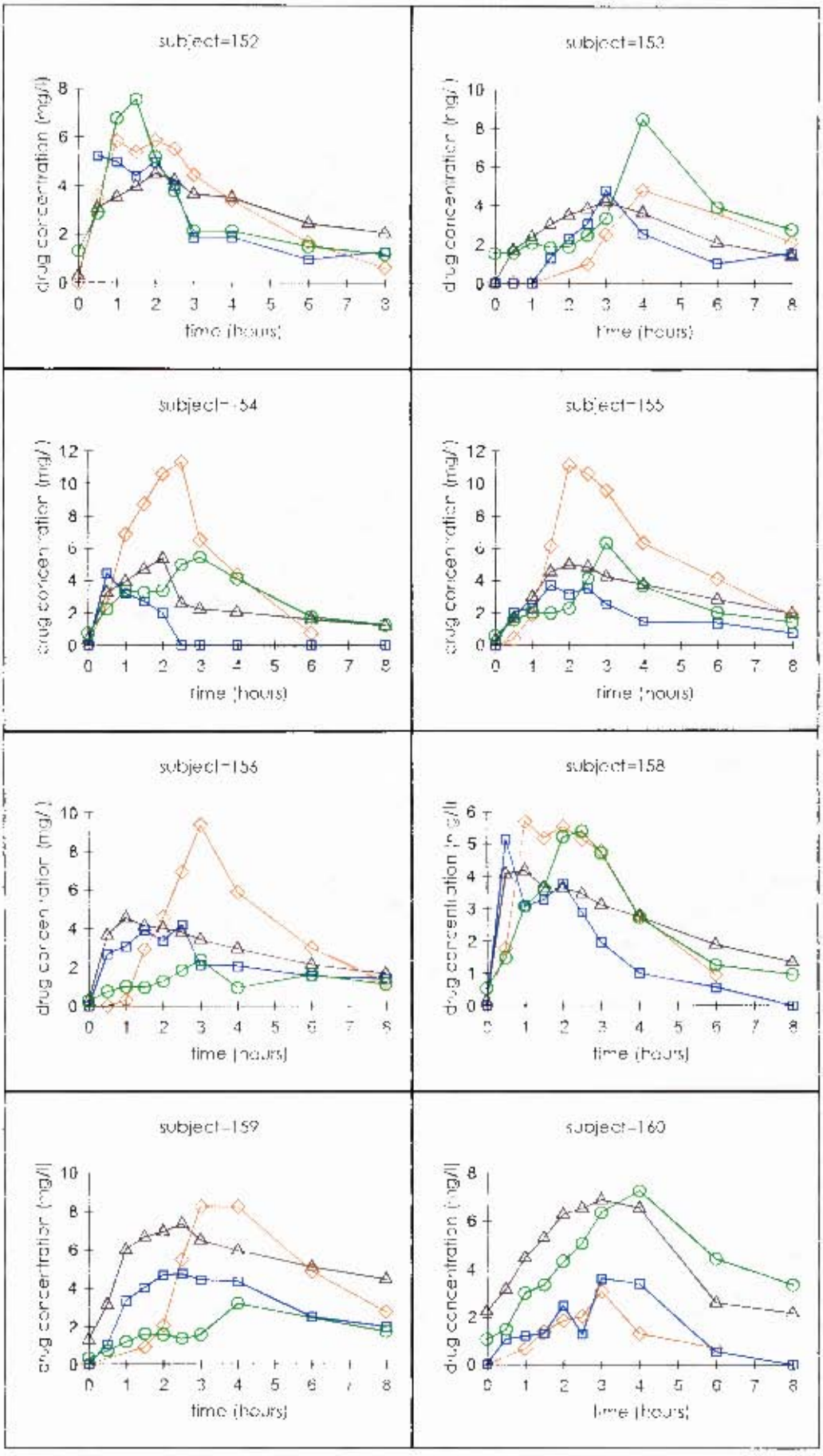
subject=145
subject=146
subject=147
subject=148
subject=149
subject=150
subject=151
drug concentration (mg/l)
time (hours)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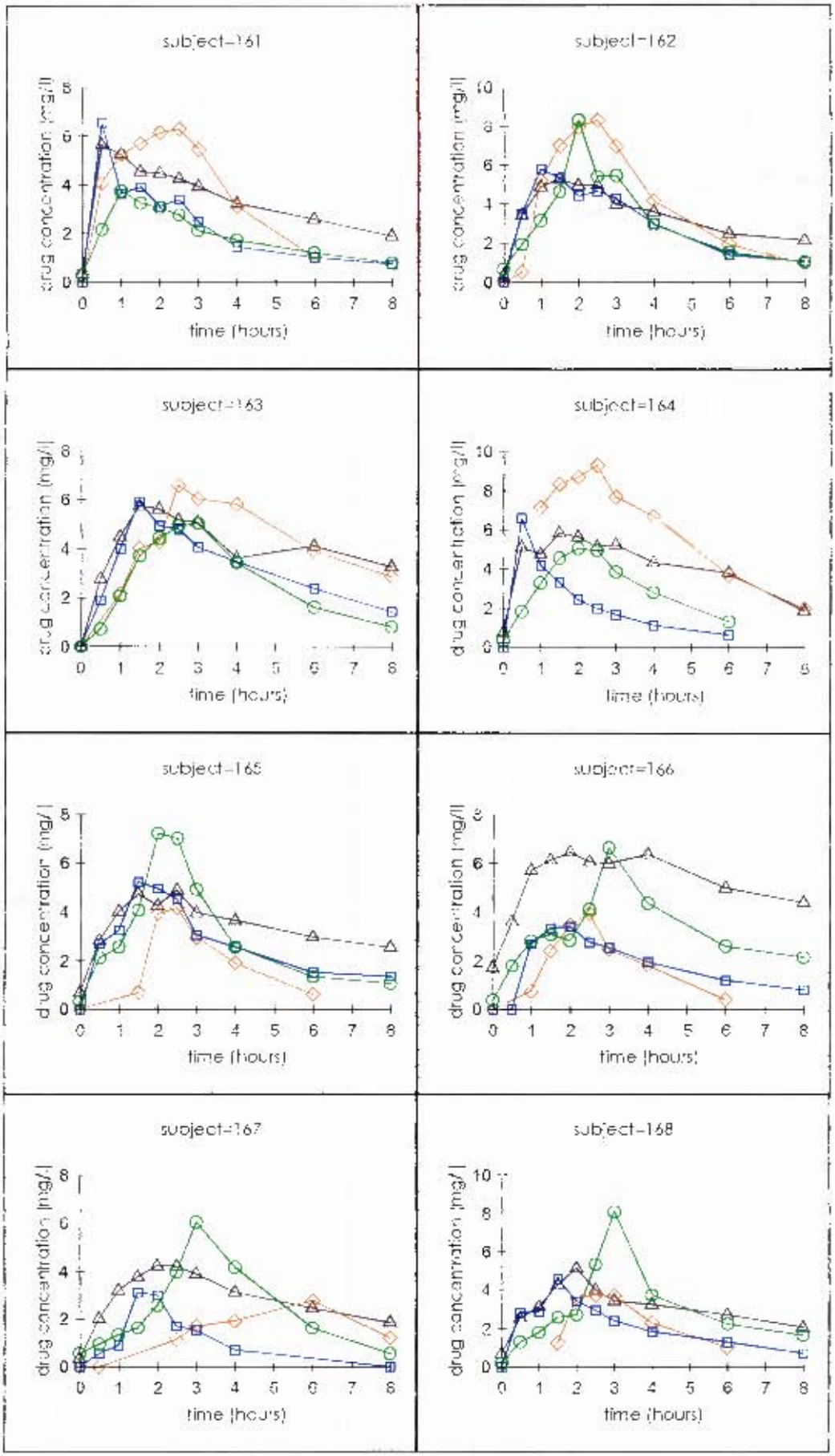
drug concentration (mg/l)
time (hours)
subject=153
drug concentration (mg/l)
time (hours)
subject=154
drug concentration (mg/l)
time (hours)
subject=155
drug concentration (mg/l)
time (hours)
subject=156
drug concentration (mg/l)
time (hours)
subject=158
drug concentration (mg/l)
time (hours)
subject=159
drug concentration (mg/l)
time (hours)
subject=160
drug concentration (mg/l)
time (hours)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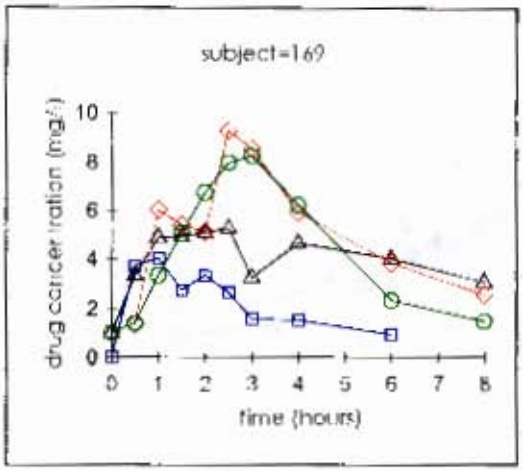
subject=162
subject=163
subject=164
subject=165
subject=166
subject=167
subject=168
drug concentration (mg/l)
time (hours)


subject=169
drug concentration (mg/L)
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
time (hours)

appendix 6

COVARIATE FACTORS FOR EACH SUBJECT

6.1

Age, sex, smoking and alcohol consumption in the year before admission, treatment category, body mass index, acetylator type, HIV-infection status and CD4+ cell count for each subject.

6.2

Chemical pathology and haematology results for each subject

6.3

Rifampicin, isoniazid, pyrazinamide and ethambutol doses per kilogram of body weight, and formulation characteristics for each subject

6.1: Age, sex, smoking and alcohol consumption in the year before admission, treatment category, body mass index, acetylator type, HIV-infection status and CD4+ cell count for each subject.

subject	Age (years)	Sex[1]	Smoking[2]	Alcohol[3]	Treatment category	Body mass index	HIV+ infection[4]	CD4+ cell count	Acetylator type[5]
1	55	[illegible]	[illegible]	1	1	20.04	0	414	1
2	27	0	0	0	1	17.30	0	533	1
3	24	0	1	1	0	17.07	0	65	[illegible]
4	33	0	0	0	0	18.32	[illegible]	23	1
5	50	1	0	0	1	19.37	0	826	[illegible]
6	26	1	0	[illegible]	1	21.96	0	836	1
7	31	1	1	[illegible]	[illegible]	15.99	1	978	1
8	36	0	1	0	[illegible]	19.82	0	1012	1
9	20	0	1	[illegible]	[illegible]	6.55	0	59	1
[illegible]	21	0	[illegible]	0	1	9.65	0	1549	1
13	19	1	0	1	1	17.37	0	432	0
14	[illegible]	[illegible]	0	0	0	20.65	1	640	0
15	72	0	0	0	1	16.47	0	504	[illegible]
16	39	0	1	1	0	24.00	1	587	1
17	56	0	1	1	1	17.75	0	896	[illegible]
18	[illegible]	0	1	1	1	25.93	0	525	[illegible]
19	36	0	1	1	1	17.44	1	583	1
20	45	1	0	2	1	17.64	1	68	0
22	31	0	1	[illegible]	0	21.37	0	461	0
23	53	1	0	0	[illegible]	25.25	0	313	1
24	22	0	0	0	0	17.10	[illegible]	139	1
25	51	0	1	[illegible]	0	6.82	1	180	1
26	32	1	[illegible]	[illegible]	1	19.11	0	1060	0
27	42	0	[illegible]	1	1	16.38	0	713	0
29	33	0	0	0	0	13.19	0	527	0
30	27	0	[illegible]	0	1	16.23	0	927	[illegible]
31	45	0	0	1	1	14.13	0	283	1
34	7	0	1	1	1	17.26	0	423	0
37	27	0	0	0	1	20.76	0	291	1
41	56	[illegible]	0	0	0	20.28	0	685	0
42	29	1	0	0	1	22.22	[illegible]	442	1
43	59	1	1	1	0	18.36	0	497	1
44	36	0	0	0	[illegible]	20.43	0	363	0
45	[illegible]	0	[illegible]	[illegible]	[illegible]	6.05	0	760	1
46	35	1	1	[illegible]	0	18.66	0	607	1
47	29	1	1	[illegible]	0	18.36	0	732	1
48	34	1	0	0	1	20.70	0	642	1
49	31	1	0	[illegible]	0	19.83	1	204	1
50	55	0	0	0	[illegible]	14.59	0	684	[illegible]
51	39	1	[illegible]	1	1	17.84	0	368	[illegible]
52	46	[illegible]	1	1	0	15.32	0	518	[illegible]
53	35	[illegible]	1	0	1	18.78	0	619	[illegible]
54	21	0	0	1	0	29.03	1	3.5	1
55	29	0	0	0	1	20.99	0	1327	1
56	33	[illegible]	1	[illegible]	1	19.56	[illegible]	220	0
57	28	0	1	1	1	18.99	0	862	1
58	31	0	1	[illegible]	[illegible]	18.59	0	1197	1
59	44	0	1	[illegible]	[illegible]	15.43	0	819	1
60	34	1	0	[illegible]	[illegible]	12.76	0	1365	[illegible]
61	47	1	1	[illegible]	[illegible]	17.15	0	605	[illegible]
63	42	[illegible]	[illegible]	0	1	17.78	0	662	1
64	58	0	0	0	0	35.59	0	299	[illegible]
65	26	0	[illegible]	0	1	17.23	0	568	[illegible]
66	44	[illegible]	1	1	1	18.00	0	1019	[illegible]
67	38	1	[illegible]	1	1	16.44	0	553	1
68	24	[illegible]	1	[illegible]	1	18.96	0	595	1
70	34	0	1	[illegible]	1	16.00	0	491	0
71	62	0	1	2	[illegible]	15.86	0	692	1
72	33	0	[illegible]	[illegible]	[illegible]	[illegible]	0	586	1
73	21	0	0	0	1	15.05	0	739	1
74	17	0	0	2	0	18.75	0	642	1

subject	Age (years)	Sex[1]	Smoking[2]	Alcohol[3]	Treatment category[4]	Body mass index	HIV-infection[5]	CD4+ cell count	Acetylatolytyle[6]
76	32	0	0	0	1	24.35	0	1370	0
77	27	1	1	1	1	16.98	0	1016	1
80	34	0	1	1	1	23.24	0	688	1
81	41	0	1	1	0	18.70	0	906	1
82	42	1	1	0	1	20.31	0	329	1
84	46	1	0	0	0	16.90	0	591	1
86	28	0	1	1	1	18.75	0	695	1
87	37	0	0	0	1	21.75	0	689	1
88	36	1	0	0	1	14.06	0	93	1
89	48	1	1	1	1	15.41	0	1231	1
90	26	1	1	0	0	17.57	1	351	1
91	28	1	0	0	1	13.29	0	988	0
92	27	1	0	0	0	22.11	1	236	1
93	45	0	1	1	0	18.70	0	702	1
94	35	0	1	1	0	16.40	0	941	1
95	42	0	1	0	0	19.43	0	1405	0
96	31	0	1	1	0	21.58	0	864	1
97	20	0	0	0	0	17.89	0	522	1
101	41	1	1	1	1	17.08	0	476	1
103	29	1	1	1	1	20.76	0	487	1
104	29	0	1	1	0	22.85	0	895	1
105	27	0	0	0	0	22.64	0	669	1
106	52	0	0	0	1	19.70	0	363	1
107	58	0	1	0	0	16.01	0		1
108	57	0	1	0	1	18.70	0	891	1
109	26	0	1	1	0	18.99	0	661	1
110	43	0	1	1	1	18.69	1	531	1
111	49	0	1	0	0	17.93	0	746	1
112	53	1	1	0	1	21.22	0	513	0
113	57	1	1	1	0	17.93	0	538	1
115	34	1	1	1	1	17.64	0		
117	21	1	0	0	1	17.97	0	-	0
118	59	0	1	0	0	22.81	0	677	-
119	60	1	1	1	1	20.76	1	707	-
120	40	1	1	1	0	16.44	0	1207	-
121	28	0	0	0	1	14.50	0	875	-
122	43	1	1	1	1	15.06	0	844	-
123	49	0	0	1	1	20.93	0	142	-
124	37	0	1	1	0	20.39	0	1122	-
125	40	0	1	1	1	21.24	0	825	
126	44	1	1	1	0	17.64	0	1237	
127	25	1	1	1	1	14.88	0	1016	-
128	51	1	1	1	0	17.63	0	1736	-
130	34	1	1	1	0	18.93	0	1498	-
131	28	1	1	0	1	19.21	1	713	
132	54	1	1	1	0	16.46	0	1317	
133	29	1	1	0	1	20.31	0	704	
134	53	0	1	1	1	14.61	0	1008	-
135	30	1	1	1	1	14.34	0	909	-
136	44	1	1	1	1	17.51	0	738	
137	37	1	1	1	1	17.65	0	340	-
138	43	1	1	1	1	16.81	0	-	
139	36	0	1	1	0	18.70	0	1339	
141	25	1	0	0	0	16.90	0	524	-
142	35	1	1	1	1	18.52	0	787	-
143	23	0	1	0	0	18.70	0	-	-
144	26	0	1	1	0	8.70	0		-
145	27	1	0	0	1	16.14	0	-	-
146	27	1	1	1	1	15.43	0		
147	35	0	1	1	1	12.86	0	-	-
148	54	0	1	1	1	16.63	0	-	-
149	37	1	1	1	1	14.36	0		
150	19	1	1	0	0	18.36	0	706	
151	30	1	1	1	1	16.09	0	674	

subject	Age (years)	Sex[1]	Smoking[2]	Alcohol[3]	Treatment category[4]	Body mass index	HIV-infection[5]	CD4+ cell count	Acetylator type[6]
152	31	1	1	1	0	16.23	0		-
153	43	0	1	0	0	13.59	0	749	-
154	24	0	1	1	1	19.07	0	827	-
155	48	0	1	1	0	20.63	0	706	
156	24	1	1	1	1	22.21	0	1285	-
158	32	0	1	1	1	20.81	0	1156	
159	28	0	0	0	0	19.33	0	562	-
160	26	1	1	0	1	17.64	0	602	
161	28	0	1	1	1	19.87	0	-	-
162	42	0	0	0	1	15.46	0		
163	32	1	0	-	1	17.64	0	-	-
164	39	0	1	0	1	25.81	0	-	
165	40	1	1	-	0	17.64	0	-	-
166	49	0		0	1	18.70	0		
167	30	0	1	1	0	28.93	0	1038	-
168	39	1	1		1	17.64	0	613	
169	29	1	1	-	1	17.64	-	654	-

[1] female=0; male=1

[2] No history of smoking in the year prior to admission=0; history of smoking in year prior to admission=1

[3] No history of regular alcohol consumption in the year prior to admission=0;
history of regular alcohol consumption in year prior to admission=1

[4] new TB patient=0; retreatment patient=1

[5] No HIV infection=0; HIV infection=1

[6] slow acetylator type=0; intermediate or rapid acetylator type=1

6.2: Chemical pathology and haematology results for each subject

subject	Total protein	albumin	Total bilirubin	ALT	AST	AP	γGT	urea	creatinine	Haem-oglobin	MCV	WCC	platelets	ESR
1	90	37	7	29	35	267	134	4.2	71	14.3	87	10.9	661	114
2	82	39	6	15	22	93	13	5.5	58	7.8	73	5.3	404	40
3	83	33	5	15	23	45	21	4.7	75	9.8	89	13.3	925	70
4	88	32	3	13	20	62	5	3.4	61	8.7	113	4	280	135
5	7	38	7	22	27	65	32	3.7	77	11.5	89	7	210	6
6	82	40	5	16	17	50	34	4	74	13.8	95	4.3	293	7
7	92	37	7	8	13	65	27	6.1	71	12.2	91	9.9	566	112
8	83	35	7	24	30	86	12	2.7	70	13.2	88	12.3	487	60
10	83	41	6	36	22	66	27	3.2	66	13.1	94	11.7	455	52
11	76	43	7	18	14	78	23	2.7	68	13.3	87	12.8	224	4
13	97	40	7	16	17	64	7	3.7	74	12.9	99	8.4	278	56
14	83	36	7	20	20	86	60	2.2	77	14.6	94	10.7	342	40
15	78	33	5	14	13	73	40	5.4	74	10.6	98	12.9	370	80
16	92	46	12	53	28	62	24	4.3	71	14.2	100	7.5	346	10
17	76	29	33	9	21	58	84	3.6	57	12	103	6.4	84	25
18	77	43	5	7	20	55	27	3.4	55	14.2	100	5.8	200	35
19	82	31	7	9	15	56	16	4.2	53	9.5	99	12.7	612	110
20	74	21	5	19	20	40	58	2.7	-	12.5	134	5.4	276	110
22	72	39	7	25	27	67	15	4.2	57	13	93	13.1	330	12
23	79	35	21	11	15	186	227	5	82	11.5	88	3.8	185	26
24	114	27	4	30	43	239	396	3	63	8.9	111	5.8	401	132
25	91	21	19	25	40	111	123	2.3	62	10.3	109	4.6	128	122
26	91	47	16	13	26	74	65	4	80	14.2	98	9.1	240	8
27	88	33	4	10	12	77	33	5.2	88	11.5	99	14.6	539	119
29	70	31	2	5	16	78	20	2.7	81	11.9	102	11.1	468	89
30	73	35	3	19	14	88	29	2.7	63	12.1	100	15.5	721	29
31	84	35	6	20	19	78	54	2.1	52	13.2	97	11.8	840	20
36	78	34	5	11	15	66	15	3.8	90	11.5	100	5.6	270	60
37	74	37	9	9	13	42	14	3.6	75	11.7	99	5	265	12
41	69	38	4	18	19	59	21	5.7	97	13.7	91	7.6	348	8
42	82	33	7	19	25	75	38	3.5	97	11.3	94	8.3	302	42
43	79	39	3	14	15	69	19	5.2	90	9.5	91	12.8	676	50
44	84	37	6	10	17	73	17	5.5	69	11.5	95	6.7	299	44
45	76	37	5	15	16	68	32	3.5	68	14.3	102	13.1	451	1
46	94	39	7	27	44	61	27	2.7	72	13.9	99	7.1	304	55
47	97	44	5	23	30	63	97	3.2	72	14.6	99	10	343	29
48	79	33	7	20	10	53	74	2.3	67	12.6	83	10	500	30
49	106	33	4	21	27	60	95	3.3	72	11.8	101	8.4	242	116
50	76	35	4	9	16	69	26	2.7	58	10.6	84	15.4	630	33
51	78	29	6	13	17	39	24	2.6	74	9.4	110	14.5	720	95
52	78	29	16	19	33	76	19	1.9	65	10	103	5.3	527	90
53	76	35	5	8	12	66	15	2.9	73	11	91	10.6	295	52
54	90	37	4	25	30	182	109	3.3	68	8.6	121	9.1	276	136
55	72	40	9	15	9	68	20	3.1	78	14.1	88	7.7	259	2
56	72	33	5	6	10	103	42	3	57	10	93	18.2	423	80
57	77	36	8	11	17	59	13	4.1	79	12.5	97	9.9	370	45
58	78	25	8	8	20	77	22	2.6	70	7.4	88	25.7	595	132
59	80	25	4	6	9	64	25	3.5	78	10	82	10.4	601	113
60	76	36	6	12	19	86	32	4.8	90	12.3	97	12.2	452	24
61	75	43	13	19	21	65	14	4.4	90	13.3	99	4.7	217	2
63	92	44	9	16	23	74	18	3	89	14.9	101	8.3	385	32
64	77	38	7	9	15	75	36	4.8	77	13.5	107	4.9	146	120
65	62	29	3	0	14	43	10	2.4	43	10.3	87	7.4	361	29
66	74	34	13	17	16	69	36	3	57	13.8	97	10.8	479	60
67	65	19	2	11	13	67	42	3.2	56	10.6	100	15.2	428	82
69	88	34	5	7	12	91	58	5.3	96	10	112	6.6	592	77
70	84	32	4	18	18	86	48	5.8	88	12.1	89	10.8	556	87
71	73	37	4	15	17	53	26	4.1	92	11.6	86	7.1	417	24
72	72	35	6	10	16	65	9	5.2	116	11.8	97	6.4	470	99
73	77	34	2	19	19	90	33	3.5	68	10.3	78	11	576	63
74	83	41	4	9	14	92	9	3.6	97	12.8	85	7	371	28
75	75	25	6	15	18	156	76	3.5	78	8.2	115	8	775	88

subject	Total protein	albumin	Total bilirubin	ALT	AST	AP	γGT	Urea	creatinine	Haem-oglobin	MCV	WCC	platelets	ESR
77	83	37	5	10	12	7	60	3.9	64	12.7	95	11.5	324	60
80	78	34	9	15	14	63	27	3.9	73	12.2	93	8.5	233	50
81	76	33	3	7	16	59	16	3.4	72	9.7	75	9.8	490	35
83	73	34	4	11	16	42	20	3.6	88	10.7	105	7.9	316	20
85	70	27	–	9	21	65	19	3.7	75	8.2	87	6.4	291	15
86	79	35	6	12	15	52	23	4.6	85	10.8	91	5.2	379	14
87	87	40	7	12	17	47	9	4.1	92	8	92	3.9	675	10
88	94	24	8	8	18	51	44	2.2	73	10.8	91	8	521	112
89	86	35	4	15	23	50	44	3.9	50	10.5	101	11.9	467	20
90	92	37	6	10	15	50	22	2.5	92	12.9	99	3.8	211	40
91	82	31	0	11	13	78	55	2.9	58	11.7	91	11	580	22
92	72	34	5	36	18	58	29	2.7	107	11.3	107	5.4	250	6
93	86	26	7	9	18	53	14	7.9	173	12.6	105	12.2	457	52
94	88	29	3	48	43	194	72	3.3	56	11.5	82	8.3	458	105
95	70	39	3	15	14	81	9	3.5	76	13.5	88	7.7	374	10
96	82	36	4	13	17	51	37	3.5	77	11.4	93	7	330	53
97	83	43	3	17	35	68	18	3.2	80	12.7	9	5.6	424	20
101	75	35	11	22	18	61	28	3.9	67	13.2	97	5.3	246	20
102	84	25	5	41	26	69	16	3.2	64	12.3	97	8	190	95
104	7	34	6	16	17	50	52	3.5	74	11.6	98	5.5	291	46
105	78	38	2	15	22	71	12	5.3	77	13.4	91	10	425	19
106	70	29	1	13	14	76	40	12.5	120	10.8	79	6.2	362	87
107	87	35	–	16	21	69	46	3.2	56	11.4	93	8.1	544	105
108	82	30	–	12	16	62	48	2	51	11.9	94	8.5	548	108
109	77	37	2	41	29	69	19	3	69	12	82	5.5	386	37
110	82	30	2	12	18	73	27	2.9	78	9.7	93	7.6	687	139
111	83	36	4	43	34	61	13	3.8	70	12.3	79	7.4	305	63
112	77	35	3	22	22	46	45	2.6	84	12.6	80	7.1	300	42
113	82	34	3	–	15	64	32	4.4	67	10.3	94	3.9	264	88
115	77	39	7	8	14	52	13	3.2	79	13.4	99	6.2	529	27
117	77	44	9	20	20	159	110	3.7	71	4.3	80	5.6	304	5
118	84	41	8	22	2	55	53	5.4	83	12.6	82	5.9	66	37
119	73	40	5	19	22	38	38	3.7	90	13.2	98	4.9	304	12
120	70	32	6	9	15	92	25	4.1	59	11.7	87	3.2	665	54
121	86	34	5	9	22	54	23	2.9	67	12.1	86	7.2	541	73
122	74	29	5	11	16	49	12	3	72	12	93	8.3	210	40
123	84	36	3	19	63	-	25	4.3	-	10.5	90	8.7	539	117
124	71	30	3	33	26	41	22	3.3	60	11.5	87	10.1	470	52
125	77	35	5	10	13	39	9	3.2	52	10.2	89	9.8	697	120
126	74	31	4	8	13	64	6	3.6	45	9.5	95	15.7	813	129
127	75	42	7	29	21	52	23	3.3	82	13.8	77	10	463	7
128	77	37	6	11	18	52	21	2.5	65	13.7	88	9	320	25
130	63	32	2	12	11	56	14	2.6	76	12.3	77	10.9	378	16
131	74	38	4	14	16	55	16	3.2	83	14.3	89	4.2	357	14
132	76	40	4	17	12	65	29	4.6	73	11.7	87	7.6	430	17
133	68	34	5	81	45	76	27	2.3	72	13	87	7.5	343	12
134	69	39	6	22.7	18.8	70.52	72.75	4.1	70	13.6	103	9.6	293	65
135	88	34	3	8.52	14.1	72.16	54.75	2.2	54	15	95	13.9	529	115
136	75	35	5	9.94	6.71	69.7	28.5	2.6	60	13.8	82	9.7	474	16
137	73	35	6	32	26.8	66.42	31.5	2.3	77	14	88	7.9	415	20
138	75	33	8	15.6	14.1	84.46	23.25	3.6	85	13.9	90	8.8	356	55
139	74	35	8	9.22	11.7	77.09	64.5	2.5	61	11.7	97	11.8	512	82
141	74	25	6	11.4	13.4	77.08	32.25	3	65	12.1	98	10.1	552	51
142	76	33	3	8.52	11.4	83.64	41.5	3.3	71	12.7	96	9.3	310	100
143	82	28	5	9.23	12.1	38.5	58.5	5.2	110	9.2	82	7.7	625	22
144	76	30	5	14.3	18.1	84.2	21	4.2	77	13.8	90	6.7	491	21
145	95	31	5	9.23	12.7	97.58	43.5	2.7	56	12.7	81	9.2	507	9
146	88	32	8	12.1	17.4	133.7	74.25	3.5	63	9.5	100	9.6	808	122
147	84	34	6	11.4	20.8	51.64	24	2.3	58	12.6	101	7.7	406	112
148	89	32	7	19.9	31.5	137.3	42.75	4.2	74	12.6	104	10	509	97
149	89	38	5	12.1	6.8	109.1	54.75	2.5	65	13	97	15.9	639	85
150	85	40	9	15.9	17.4	97	45.5	3	87	14.3	79	8.2	276	10
151	69	35	8	22	24.1	61.5	20.5	2.9	82	12.8	98	8	324	16
152	88	33	5	10.7	10.7	85.6	57.75	2.9	92	12.8	89	6.1	414	40

subject	Total protein	albumin	Total bilirubin	ALT	AST	AP	γGT	urea	creatinine	Haem-oglobin	MCV	WBC	platelets	ESR
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
154	75	35	9	33.4	37.5	69.7	33	4.1	80	11.2	80	10.4	402	13
155	[illegible]	34	6	14.9	15.4	82	13.5	5.1	91	10.7	106	4.9	205	70
156	84	36	6	13.5	17.4	64.78	18.75	4	84	11.7	80	7.7	544	11
[illegible]	[illegible]	[illegible]	[illegible]	14.9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
159	80	29	12	10.7	13.4	70.57	15.75	4.5	76	11.8	89	7.4	346	27
160	82	26	7	19.2	23.5	107.4	30.75	4.4	71	9.8	87	13.9	552	27
161	79	36	4	9.29	15.4	74.67	12	2.9	56	12.2	92	8.5	602	50
162	93	36	5	9.94	17.4	104.9	67.25	2.8	75	[illegible]	84	13.6	558	16
163	71	23	12	19.9	18.6	226.3	94.5	3.7	60	10	101	12.1	544	30
64	65	36	8	7.8	7.37	94.22	35.25	4.3	67	13.7	78	7.2	440	80
[illegible]	79	33	9	22.2	19.4	101.7	45.5	2.2	67	12.2	93	15.3	519	40
166	70	25	6	11.4	18.1	121.4	40.5	2.5	56	10.2	100	17.5	293	-
167	76	39	3	41.2	22.1	59.86	37.5	2.7	72	13.4	87	6.1	317	-
168	64	33	7	15.6	10.7	53.3	30.75	2.8	86	13	87	7.3	356	-
169	80	32	3	14.9	16.8	79.54	18.75	2.2	68	13.1	79	6.7	324	-

6.3: Rifampicin, Isoniazid, pyrazinamide and ethambutol doses per kilogram of body weight, and formulation characteristics for each subject

subject	Rifampicin mg/kg	Isoniazid mg/kg	Pyrazinamide mg/kg	Ethambutol mg/kg	Formulation type¹	Formulation regulatory Status
1	9.98	6.65	33.26	17.74	0	0
2	10.42	6.94	34.72	27.78	0	0
3	11.87	7.92	39.58	0.00	0	0
4	9.59	6.40	31.32	17.06	0	0
5	12.00	8.00	40.00	20.00	0	0
6	8.82	4.41	29.4	17.65	0	0
7	9.78	6.52	32.6	26.09	0	0
8	12.93	6.47	32.33	25.86	0	0
9	9.28	6.19	30.93	24.74	0	0
10	9.78	6.52	32.61	26.09	0	0
11	9.00	6.00	30.00	24.00	0	0
12	9.03	6.02	30.17	18.10	0	0
13	12.82	8.55	42.74	34.19	0	0
14	8.57	5.71	28.57	22.86	0	0
15	11.47	7.61	38.07	20.30	0	0
16	9.38	6.25	31.25	16.67	0	0
17	11.78	7.85	39.27	0.00	0	0
20	-	-	-	-	0	0
22	10.83	5.42	36.10	0.00	0	0
23	10.91	5.45	36.36	21.62	0	0
24	11.39	7.59	37.97	30.36	0	1
25	10.71	7.14	35.71	28.57	0	1
26	11.54	5.77	38.46	15.38	0	0
27	10.87	7.25	36.23	19.32	0	1
28	15.79	10.53	52.63	28.07	0	1
30	12.00	8.00	40.00	21.33	0	1
31	14.71	9.80	49.02	26.14	0	1
34	11.14	7.43	37.13	19.80	0	1
37	9.76	6.51	32.54	26.03	0	1
41	10.00	5.00	33.33	20.00	0	0
42	9.00	6.00	30.00	24.00	0	1
43	9.57	6.38	31.91	25.53	0	1
44	9.53	6.36	31.78	16.95	0	1
45	12.10	8.06	40.32	32.26	0	1
46	9.78	6.52	32.61	26.09	0	1
47	9.57	6.38	31.91	25.53	0	1
48	11.32	5.66	37.74	22.64	0	0
49	11.11	5.56	37.04	22.22	0	0
50	14.47	9.65	48.23	25.72	0	1
51	11.1	5.56	37.04	22.22	0	0
52	9.78	6.52	32.61	26.09	0	1
53	11.32	5.66	37.74	22.64	0	0
54	9.47	6.32	31.58	25.26	0	1
55	9.66	6.44	32.19	14.7	0	1
56	12.20	10.00	40.00	26.67	0	1
57	11.08	7.39	36.95	22.56	0	1
58	12.03	8.02	40.11	21.39	0	1
59	11.54	7.69	38.46	30.77	0	1
60	12.50	8.33	41.67	33.33	0	1
61	10.00	6.67	33.33	25.67	0	1
63	11.54	5.77	38.46	23.08	0	0
64	7.02	3.51	23.39	14.04	0	0
65	11.3	7.54	37.69	20.10	0	1
66	12.24	6.12	40.82	24.49	0	0
67	11.25	7.50	37.50	30.00	0	1
68	11.84	7.89	39.47	23.68	0	1
70	10.71	7.14	35.71	28.57	0	1
71	10.50	6.84	24.21	29.06	0	1
72	8.86	5.91	19.69	23.62	0	1
73	12.61	8.40	42.02	33.61	0	1
74	10.25	6.83	34.17	27.33	0	1
75	11.00	7.33	36.67	29.34	0	1
77	11.54	5.77	38.46	23.08	0	0
80	10.08	6.04	25.21	13.45	0	0

subject	Rifampicin mg/kg	Isoniazid mg/kg	Pyrazinamide mg/kg	Ethambutol mg/kg	Formulation type¹	Formulation regulatory status
81	1.58	5.73	28.96	22.17	0	0
83	11.54	5.77	38.46	23.08	0	0
87	9.38	6.52	32.61	0.00	0	1
86	11.22	7.48	37.41	19.95	0	1
87	9.18	6.12	30.61	24.49	0	1
88	12.50	8.33	41.67	33.33	0	1
89	10.23	6.82	34.09	27.27	0	1
90	12.24	6.12	40.82	24.49	0	0
91	12.00	8.00	40.00	32.00	0	1
92	8.76	4.38	29.20	17.52	0	0
93	12.61	8.40	42.02	22.41	0	1
94	13.24	8.82	44.12	25.50	0	1
95	9.28	6.19	30.93	24.74	0	1
96	9.85	4.93	32.84	19.70	0	0
97	9.89	6.55	32.75	26.20	0	1
10.	12.90	9.40	43.00	18.81	2	0
103	10.00	5.00	33.33	20.00	0	0
104	11.38	5.69	28.41	22.75	0	0
105	8.38	5.59	27.93	0.00	0	1
106					0	1
107	12.39	8.27	41.10	0.00	0	1
108	10.37	6.91	34.56	18.43	0	1
109	9.42	6.41	32.05	25.64	0	1
110	10.84	7.23	36.14	19.28	0	1
111	10.98	7.32	36.59	24.39	0	1
112	8.82	4.41	29.41	22.06	0	0
113	12.00	8.00	40.00	24.00	2	0
115	9.18	6.12	30.61	24.49	0	1
117	9.78	6.52	32.61	26.09	0	1
118	9.66	4.83	24.15	12.88	0	0
119	9.68	4.84	32.26	19.35	0	0
120	12.16	8.11	40.54	27.03	1	0
121	10.74	7.16	35.80	23.87	1	0
122	10.71	7.14	35.71	20.97	1	0
123	9.55	6.37	31.83	21.23	1	0
124	9.66	6.45	32.26	23.81	1	0
125	9.41	6.28	31.38	20.92	1	0
126					1	0
127	0.71	7.14	35.71	28.57	1	0
128	12.50	8.33	41.67	25.00	0	0
130	8.04	5.36	26.79	0.00	1	0
131	9.52	6.34	31.71	7.14	1	0
132	8.82	5.88	29.41	9.61	1	0
133	10.34	7.76	34.48	0.00	0	0
134	12.82	8.55	28.49	23.68	0	0
135	11.25	7.50	37.50	30.00	1	0
136	11.32	5.66	37.74	22.64	0	0
137	8.82	5.88	29.41	23.53	1	0
138	12.50	8.33	41.67	25.00	0	0
139	12.64	8.43	42.13	28.09	1	0
141	13.04	6.52	43.48	0.00	0	0
142	9.38	6.25	31.25	25.00	2	2
143	2.50	8.33	27.78	27.78	1	0
144	10.64	7.09	23.66	23.64	1	0
145	10.00	6.67	33.33	26.67	0	0
146	15.00	7.50	66.67	30.00	2	0
147	14.20	9.46	47.32	31.55	1	0
148	10.44	6.96	34.80	27.84	1	0
149	10.98	7.32	36.59	29.27	1	0
150	11.32	5.66	37.74	22.64	0	0
151	10.79	7.19	35.97	28.78	0	1
152	10.71	7.14	35.71	28.57	0	1
153	14.93	9.95	47.77	31.85	1	2
154	11.54	7.69	38.46	25.64	1	0
155	11.65	5.83	38.83	23.30	0	0
156	11.54	5.77	28.85	19.23	0	0

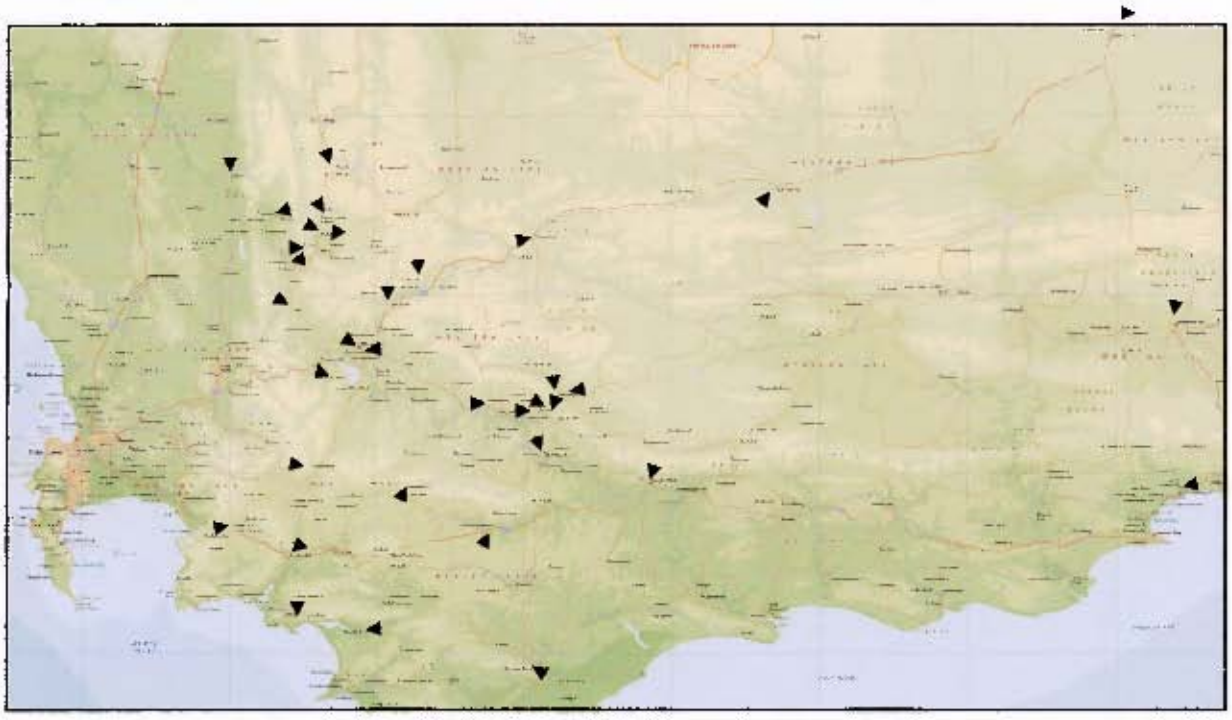
subject	Rifampicin mg/kg	Isoniazid mg/kg	Pyrazinamide mg/kg	Ethambutol mg/kg	Formulation type[1]	Formulation regulatory Status[2]
158	12.00	6.00	90.00	20.00	1	0
159	11.11	5.56	37.04	22.22	1	0
160	12.82	8.55	42.74	34.19	1	0
161	11.39	7.59	37.97	25.32	1	0
162	11.66	7.77	38.66	25.91	1	0
163	13.40	8.26	41.32	33.06	1	0
164	9.68	4.84	32.26	19.35	1	0
165	9.38	6.25	31.25	25.00	1	0
166	7.86	8.57	42.86	28.57	1	0
167	8.52	4.26	28.41	17.05	1	0
168	6.63	5.81	38.76	23.26	1	0
169	10.23	6.82	34.09	27.27	1	0

[1] applicable to rifamycin and isoniazid only; single drug formulation=0; fixed dose combination=1
all pyrazinamide and ethambutol doses taken on the day of pharmacokinetic sampling were in single drug formulations
[2] applicable to rifampicin only; approved formulations=0; formulations not approved by the regulatory authority=1

appendix 7

STUDY PATIENT TB CLINICS

The geographical distribution of TB clinics where study patients were referred for treatment after discharge from Brewelskloof Hospital.



CLINIC	Number of study patients
Beaufort West	1
Bree River	5
Bella Vista	8
Hamlet	3
Koue Bokkeveld	2
Nduli	1
Tulbach	1
Warm Bokkeveld	2
Genadendal	1
Botrivier	1
Bredasdorp	1
Grabouw CHC	11
Zwelihle	3
Stanford	3
Villiersdorp	10
Groot Brak	1
Lamsburg	2

CLINIC	Number of study patients
Oudtshoorn	1
Ashton-Kogmanskloof	2
Ashton	6
Rawsonville	1
Ashton-Zolani	2
Barrydale	6
Montagu	11
Robertson	13
Saron	5
Swellendam	7
Riviersonderend	1
CHC	6
De Doorns	6
Wolseley	4
Sanchis	9
Touwsrivier	3
Zwelethemba	3

appendix 8

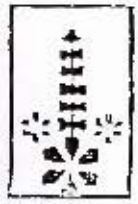
PRODUCT BATCHES NOT APPROVED BY THE NATIONAL REGULATORY AUTHORITY (THE MEDICINES CONTROL COUNCIL)

8.1

Notice of recall of product batches not approved by the regulatory authority

8.2

Comparative dissolution testing data for products not approved by the regulatory authority



LUPIN

LABORATORIES South Africa (Pty.) Limited

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Fax : 0927 – 58 - 303 5487

21 February 2000

Dear Dispenser / Sir / Madam

URGENT MEDICINE RECALL - CLASS 1, TYPE B

Product	Registration Numbers	Lot Numbers
Rifacap 150	29/20.2.3/0060	908074 + 908075
Rifacap 450	29/20.2.3/0061	908075 + 908077

We have been informed by the Medicines Control Council to recall the following two products from the market place. There was a change in the excipients added to the formulation and as a result the products could possibly pose a health hazard to the patient.

Please remove all stock of the abovementioned products from your shelves and return to Lupin Laboratories South Africa (Pty) Limited, c/o Quatromed Limited, 10 Lindley Street, Bethlehem 9701 at our expense or contact us at 058-3035476 for uplifment of any such stock.

RIFAMPICIN

COMPARATIVE DISSOLUTION TEST

The test was done according to the method described in the USP XXII, using the stainless steel basket, in three different media namely 0,1 N HCL, water and phosphate buffer pH 8,5. The dissolution media volume was 900ml and the time 45 minutes.

ANALYSIS

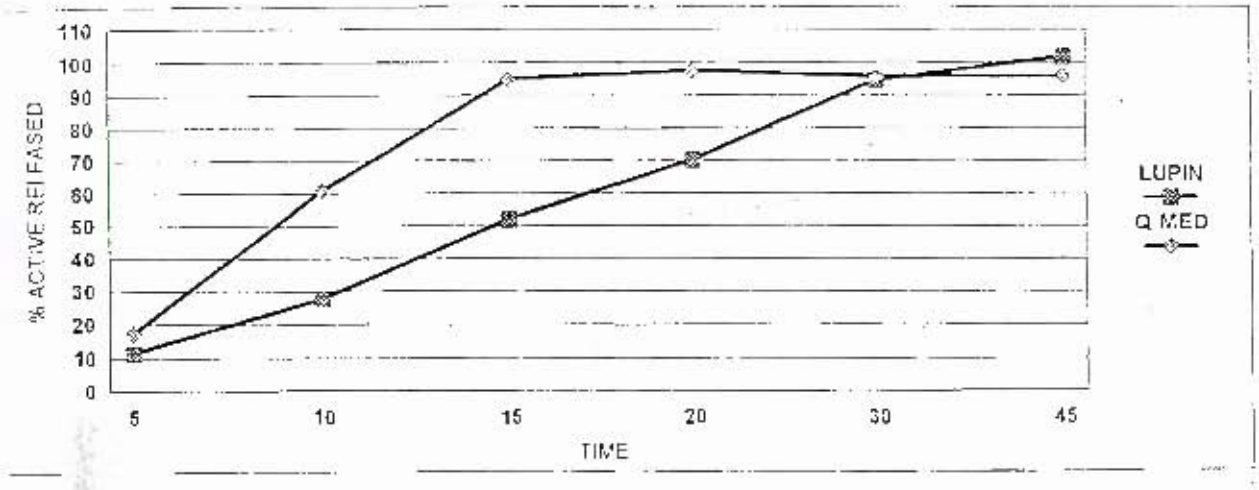
Samples (10ml) were drawn manually and filtered, 5ml diluted to 25ml (150mg) and 1ml - 25 (450mg) with dissolution media and analysed with a spectrophotometer, measuring the absorbance at 475nm

A standard graph was drawn up in all the different dissolution media. The standard graph ranged from 0,1mg to 0,7mg and was found to be linear.

A. Yberna
LAB MANAGER

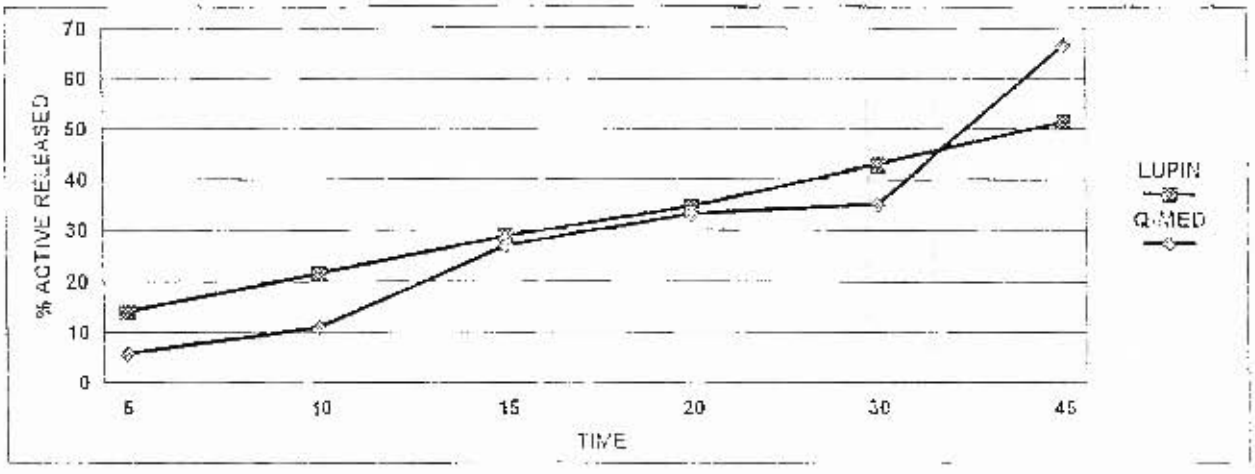
EDIUM:	0,1 N HCL	BATCH NO: 908076		450mg	[LUPIN]			
Time	Capsule no.						MEAN	STANDARD
inutes	1	2	3	4	5	6		DEVIATION
5	6.95	7.36	15.18	9.66	17.02	13.34	11.42	4.05
10	19.32	7.36	39.56	31.74	34.5	32.66	27.52	10.89
15	37.26	53.82	59.8	52.9	57.5	49.68	51.82	7.28
20	62.1	66.54	78.66	73.14	72.68	66.7	70.3	5.27
30	90.16	97.52	100.74	91.54	95.65	92.92	94.76	3.63
45	100.28	104.86	100.28	99.36	102.12	103.5	101.73	1.96

EDIUM:	0,1 N HCL	BATCH NO: 903205		450mg	[Q-MED]			
Time	Capsule no.						MEAN	STANDARD
inutes	1	2	3	4	5	6		DEVIATION
5	7.82	13.8	14.72	15.18	18.86	32.2	17.09	7.5
10	46.46	55.2	57.5	55.2	66.7	84.18	60.87	11.98
15	100.28	94.76	91.08	97.52	93.84	91.54	94.83	3.24
20	95.68	94.76	97.98	100.74	100.28	95.68	97.52	2.33
30	95.68	95.68	95.68	100.28	95.68	91.54	95.75	2.53
45	97.98	92	95.68	102.12	95.68	93.38	96.14	3.27



MEDIUM:	H2O	BATCH NO: 908076		450mg	[LUPIN]			
Time Minutes	Capsule no.						MEAN	STANDARD DEVIATION
	1	2	3	4	5	6		
5	19.78	11.04	11.04	1.84	4.14	4.14	14.03	6.09
10	32.2	17.48	20.24	5.98	44.62	6.74	21.54	13.36
15	42.78	24.38	26.9	9.66	51.98	13.34	28.67	15.05
20	49.22	32.2	37.26	12.88	57.5	17.48	34.42	15.89
30	57.96	38.64	49.22	17.48	65.32	26.52	42.89	16.54
45	65.78	50.14	60.26	20.7	69.46	41.86	51.36	16.57

MEDIUM:	H2O	BATCH NO: 903205		450mg	[Q-MED]			
Time Minutes	Capsule no.						MEAN	STANDARD DEVIATION
	1	2	3	4	5	6		
5	9.2	5.44	1.84	7.36	8.28	1.84	5.82	2.94
10	9.2	7.36	11.5	25.76	9.2	2.76	10.96	7.14
15	14.72	24.84	23.46	29.06	29.9	40.48	28.91	7.74
20	24.38	34.5	31.28	28.52	35.88	44.16	33.12	6.22
30	37.26	41.86	38.18	34.04	33.58	59.34	35.04	8.78
45	53.82	74.52	68.54	65.78	54.74	81.88	66.54	10.04



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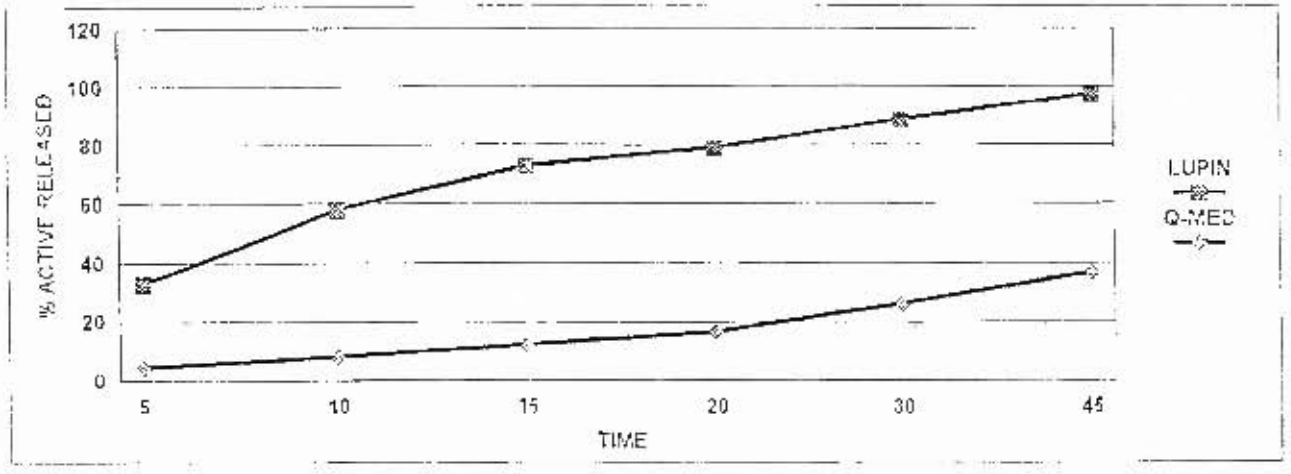
A. Yiema
LAB MANAGER

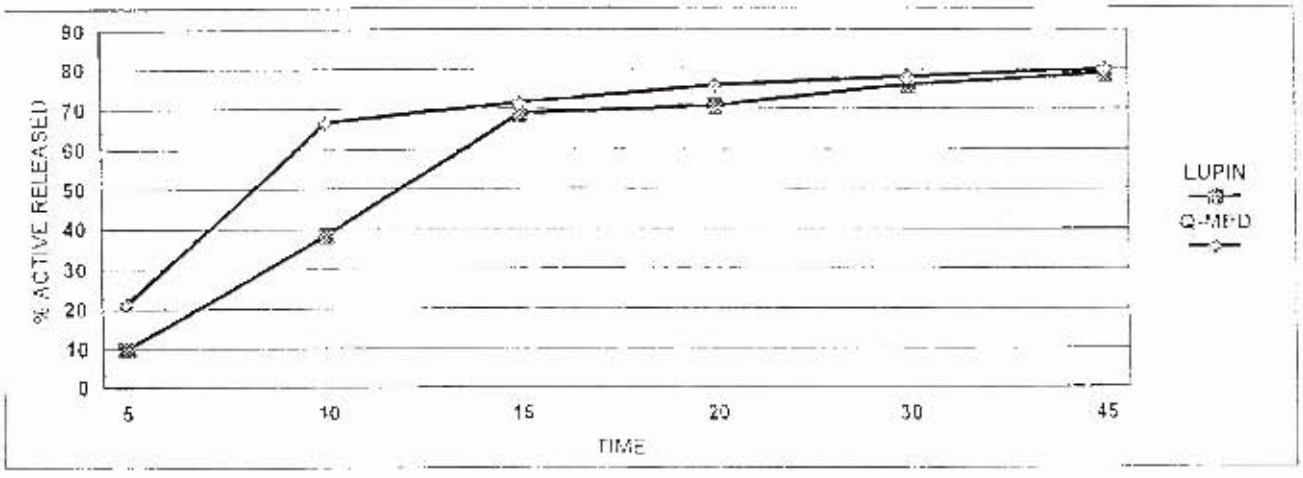
MEDIUM:	H2O	BATCH NO: 908074			150mg	[LUPIN]		
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	25.11	44.16	25.66	25.39	43.6	32.29	32.7	8.28
10	58.78	63.75	43.6	56.65	75.9	48.55	57.95	10.38
15	65.41	73.14	56.02	74.24	90.52	76.72	72.67	10.55
20	71.48	75.34	69	80.59	95.77	81.42	78.93	8.75
30	77	80.31	87.21	89.32	108.74	90.8	88.73	10.13
45	82.24	87.21	95.77	94.94	117.57	105.96	97.28	11.71

MEDIUM:	H2O	BATCH NO: 903203			150mg	[Q-MED]		
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	3.03	7.45	4.14	1.93	2.48	5.79	4.13	1.94
10	3.31	10.48	7.45	3.86	5.52	17.39	8	4.83
15	7.17	17.86	16.83	6.9	7.45	22.35	12.09	5.79
20	9.66	14.07	25.66	9.93	8.83	30.36	16.41	8.47
30	17.94	18.21	45.5	18.49	11.31	44.98	26.07	13.77
45	26.77	28.42	65.41	27.6	14.62	58.23	36.84	18.37



MEDIUM:	0.1 N HCL	BATCH NO: 908074		150mg	[LUPIN]			
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	2.76	33.67	4.41	2.76	3.03	12.42	9.84	11.19
10	21.52	57.4	27.32	22.9	50.23	50.78	38.35	14.73
15	59.06	77.55	59.34	60.44	78.38	76.1	68.81	9.21
20	72.31	69.27	68.72	71.76	71.48	70.1	70.6	1.33
30	73.96	72.86	74.24	80.31	78.66	75.34	75.59	2.69
45	82.5	77.28	77.28	80.99	78.66	75.34	78.73	2.9

MEDIUM:	0.1 N HCL	BATCH NO: 903203		150mg	[Q-MED]			
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	29.53	22.06	20.14	4.96	16.28	33.39	21.06	9.2
10	67.62	68.44	65.13	70.65	63.2	64.03	66.51	2.62
15	78.93	69	71.48	71.03	65.96	72.31	71.45	3.94
20	78.99	79.21	52.04	73.96	73.14	76.45	75.63	3.11
30	83.62	82.8	72.31	75.62	75.07	77.83	77.88	4.11
45	83.93	83.23	77	78.93	75.9	80.31	79.88	2.97



MEDIUM:	BUFFER	BATCH NO: 908074		150mg	[LUPIN]			
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	18.21	26.42	51.33	26.42	60.59	46.52	42.31	20.56
10	53.82	88.32	107.64	99.08	121.16	107.91	96.32	21.44
15	73.41	111.78	123.92	121.71	125.3	128.13	113.7	18.64
20	101.84	124.2	132.2	136.06	138	135	128.38	12.45
30	128.34	136.89	141.31	142.14	138	141.31	137.99	4.72
45	141.58	141.31	148.21	146	146.55	142.96	144.43	2.62

MEDIUM:	BUFFER	BATCH NO: 903203		150mg	[Q-MED]			
Time	Capsule no.						MEAN	STANDARD
Minutes	1	2	3	4	5	6		DEVIATION
5	27.6	32	3.66	2.2	2.2	3.31	11.81	12.79
10	54.64	49.4	6.34	4.59	5.24	6.62	21.15	21.89
15	88.32	86.17	9.1	12.97	9.93	10.21	33.11	32.46
20	107.64	84.18	11.59	17.11	14.62	15.73	41.81	36.88
30	128.61	111.5	16.83	27.6	28.98	24.01	56.26	45.55
45	136.82	129.99	22.35	43.88	47.74	34.77	69.22	46.05